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13. ABSTRACT *(Maximum 200 Words)* This report documents progress made in the third year of DAMD17-96-1-6097 "Role of seprase in breast cancer invasion". The goals are to clone a full length cDNA encoding seprase and then produce cells that overexpress seprase to investigate its role in tumor cell invasion. A chicken embryo seprase cDNA has been used to produce a seprase-calmodulin binding protein fusion protein that will be used to produce new antibodies to seprase. Full-length (2.4 kb) and truncated (1.1 kb) seprase cDNAs have been cloned from MDA-MB-436 human breast cancer cells and characterized. Seprase over-expression by breast cancer cells has been directly demonstrated biochemically and with seprase-specific cDNA probes. Moreover, we have demonstrated that MDA-MB-436 cells express active seprase. MDA-MB-436 cells that are down-regulated with respect to cell-surface seprase expression have been produced by transfection with seprase DNA expressed in the antisense orientation. Two papers describing new results regarding this project were published this year. The research progress is on time with respect to the original statement of work and we expect successful completion of the proposed research within the 4 year project period.

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Thomas J. Kelly, Jr. 10/7/99

PI - Signature Date

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Introduction

This report describes progress made in the third year of a four year project entitled "Role of seprase in breast cancer invasion." Seprase is a serine integral membrane protease (1) first identified in human melanoma cells (2) and chicken embryo fibroblasts transformed by Rous sarcoma virus (3). In 1997, a cDNA encoding human melanoma seprase was cloned and sequenced by others (1). The sequence reveals that seprase is a member of a family serine integral membrane proteases that includes fibroblast activation protein-α (FAP-α) (4), and dipeptidyl peptidase IV (DPPIV) (5, 6). Seprase is known to degrade gelatin (1-3) and is thought to facilitate erosion of the extracellular matrix thereby promoting invasion of malignant cells. Supporting this concept is the association of seprase overexpression with the invasive phenotype and its concentration on the invadopodial membranes of human melanoma cells (2, 7). Moreover, accumulation of seprase on the surface of invadopodial membranes is stimulated by ligation of $\alpha_6\beta_1$ integrin on the melanoma cell surface (8). The localization of seprase to invadopodia supports a role for seprase in degrading extracellular matrix and facilitating tumor cell invasion because invadopodia are specialized protrusions of the plasma membranes of invasive cells that contact and degrade extracellular matrix (9, 10). Invadopodia can cause proteolysis of intact fibroblast extracellular matrices, type I collagen, type IV collagen, laminin, and fibronectin (3). Several malignant cell types are known to use invadopodia to degrade extracellular matrix including, human melanoma cells (2, 7, 10), Rous sarcoma virus-transformed chicken embryo fibroblasts (3, 9, 11) and human breast cancer cells (12-14). The fact that malignant human breast cells degrade extracellular matrix with invadopodia suggested that seprase might have a role in promoting the invasive spread of human breast cancer.

Seprase is overexpressed by invasive human breast cancer (15, included in the appendix). This conclusion is now based on four lines of evidence three of which have been published and a fourth being prepared for publication as the direct result of the funding of this project. Specifically, a seprase-specific polyclonal antibody produced using affinity-purified chicken embryo seprase intensely labels malignant breast cells but not normal breast epithelia or stromal cells (15). In addition, seprase activity was detected by zymography in extracts of human breast cancer tumors and the seprase activity was five times greater than that of chicken embryo seprase as determined using a ^{3}H-gelatin substrate (16, included in the appendix). Seprase activity was detected by zymography in extracts of human breast cancer cell lines but not in extracts of a normal human breast cell line. Moreover, with this report we provide direct evidence for the over expression of seprase by invasive human breast cancer cells. Thus we believe that seprase has an important role in breast cancer cell invasion. To investigate the role of seprase in breast cancer cell invasion we proposed these specific aims:

1) Identify and characterize a full-length cDNA for human breast cancer seprase.

2) Investigate the contribution of seprase to the metastatic potential of breast cells.

Progress made towards completing these objectives is described in the body of this report in relation to the statement of work submitted with the original proposal.

Body

Personnel changes:

Hailing (Helen) Zhang, graduate student in the Department of Pathology, completed her Master's degree and has left the laboratory.

Johnna D. Goodman has joined the laboratory to pursue her Ph.D. on this project in the Pathophysiology program. Johnna is leading the effort to produce transfectants of MDA-MB-436 breast cancer cells that are down-regulated in seprase activity. Johnna's student stipend is not supported by the DoD monies.

Other personnel:

Tricia Rozypal is a Research Technologist II who devotes 100 % of her effort towards this project. Ms. Rozypal has cloned and sequenced human breast cancer seprase and is now engineering MCF-7 human breast cancer cells to over-express seprase. Ms. Rozypal's entire salary and benefits are provided by the DoD monies.

Thomas Kelly, Ph.D., is the PI on the project and continues to devote 60 % of his time towards data analysis and interpretation, deciding experimental strategies, writing and publishing the findings, and performing experiments pertinent to this project. The DoD monies provide 60 % of Dr. Kelly's salary and fringe benefits.

Progress in year 3

SOW Task 1. Months 1-3: Produce cDNA expression library with mRNA

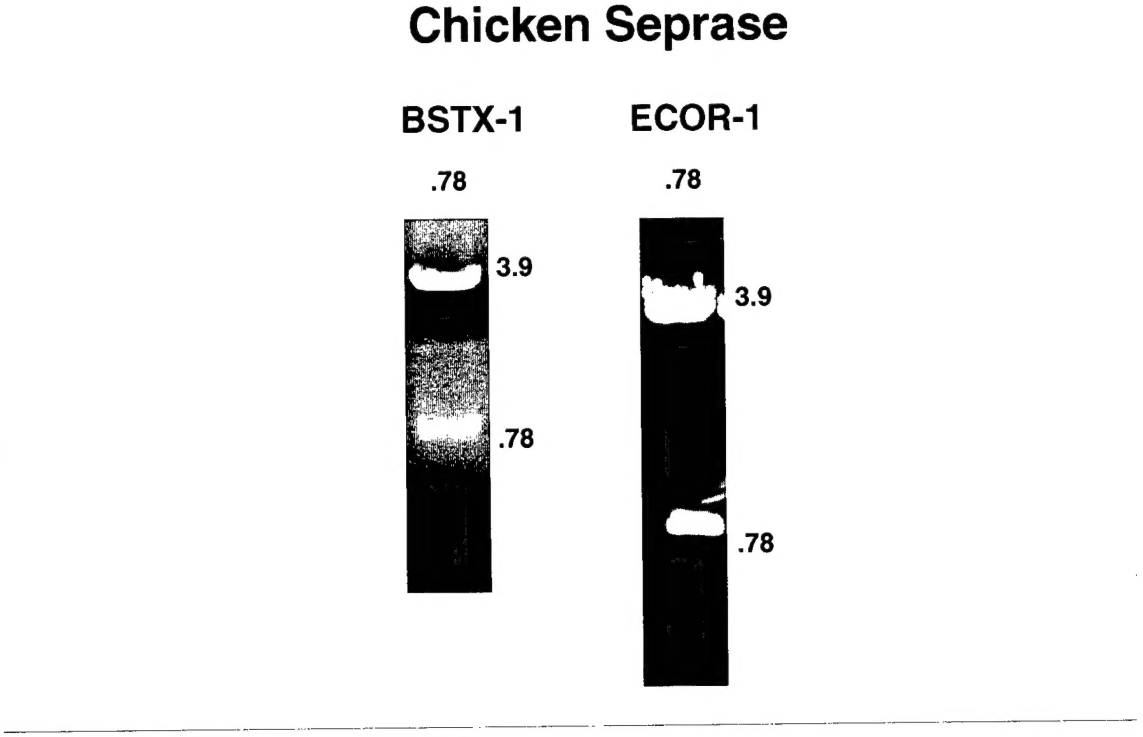

Figure 1. Restriction enzyme digestion of the partial chicken seprase cDNA. Ethidium bromide stained agarose gel revealing 3.9 kb and 0.78 kb restriction fragments generated by digestion of the pCR2.1 vector from clone 7 with BSTX-1 and ECOR-1 (left & right panels respectively). These fragments correspond to the predicted sizes of the plasmid (3.9 kb) and the partial chicken embryo seprase cDNA (0.78 kb).

purified from human breast tumors.

As discussed in earlier reports, we have already produced high quality chicken embryo cDNA expression libraries. We have focused our efforts on RT-PCR cloning of the human breast cancer seprase cDNA from the breast cancer cell line MDA-MB-436 as described below.

SOW Task 2. Months 1-36: Produce additional seprase-specific probes for screening the cDNA expression library.

I) Oligonucleotide probes based on seprase, fibroblast activating protein-α (FAP-α) and dipeptidyl peptidase IV (DPPIV) sequences. We have now cloned seprase cDNAs (described below) and have used these to produce seprase specific oligo-deoxynucleotide primers to sequence the DNA. Many of these primers were determined by analyzing the seprase sequences we obtained with the GCG sequence analysis programs. (Primers are given in order of appearance in the assembled sequence):

C-Terminal Chicken Seprase Nucleic Acid Sequence

```
        1472                                GAAAGGTGC CAGTATTATT
CAGCGAGGTT CAGTGAACGC TCTAAGTATT ATGCCTTGAT CTGTTATGGT
CCCGGGATTC CTATTTCTAC TCTTTTTGAG AATGAGAGTG ATAGAGAGCT
CAGAATATTA GAGGACAATC AAGAATTGCA GTCTGCTTTG CAAGAGATCA
TACTGCCAAA AGAAGAAATT AATAAACTTG AAGTGGATGG TATAACTTTG
TGGTACAAAA TGCTTATACC CCCACAATTT GATAGATCCA AGAAGTACCC
ACTGCTTATT CAAGTGTATG GAGGACCATG CAGTCAGAAT GTAAAACACA
CATTTAGCAT TAGCTGGATA ACGTATCTTG CAAGCAAAGA GGGAATTATT
GTTGCTCTAG TAGATGGCAG AGGAACAGCT TATCAAGGTG ACAAGATTTT
GCATGCAGTT TATCGAAGAC TAGGAGTCTA TGAAGTTGAG GACCAAATTT
CAGCCGTGAA GAAATTTATA GAAATGGGTT TTATTGATGA GAAACGAATA
GCAATATGGG GCTGGTCCTA TGGTGGATAT GTAACTTCTC TGGCACTTGG
ATCTGGCAGT GGAGTATTTA AATGTGGAAT AGCTGTGGCT CCTGTTTCCA
GTTGGGAATA TTACGCATCT ATCTACACAG AACGATTTAT GGGTCTTCCT
GTAGAGTCCG ATAATCTTGA GCACTATAAG AATTCAACTG TGATGGCAAG
AGCAAAGAAT TTCCAAAACG TTGAGTATCT TCTCATTCAT GGAACAGCAG
ATGATAATGT              2252
```

Figure 2. Nucleic acid sequence of the 0.78 kb C-terminal chicken seprase. The numbers before and after the sequence correspond to the nucleic acid position in the published human melanoma seprase sequence that is 2366 nucleic acids in length. Purple nucleic acids are different from human seprase sequence and black nucleic acids are identical to human seprase.

M13 Forward: 5'-GTA AAA CGA CGG CCA G-3'
Primer 2: 5'-AGG AAA TGA GCT TCC TCG TCC A-3'
Primer 9: 5'-TGT GAC TTC AGG GAA GAC TGG C-3'
Primer 7: 5'-TTG CCT GGA AAT CCA CTT GTG C-3' (Sequence obtained with this primer was reversed for assembly in the sense (5' to 3') direction)
Primer 1: 5'-CGA GGA ACA GCT TTC CCA AGG T-3'

Primers were also produced for to obtain sequences that overlapped with those produced by the listed primers. These were used to confirm sequences and to determine the rare mistakes by the automated sequencing. These primers were:

M13 Reverse: 5'-CAG GAA ACA GCT ATG AC-3'
Primer 5: 5'-ATC CAC CAA GGC AAT GAC-3'
Primer 6: 5'-TGT TCC AGC AAT GAT AGC C-3'

Finally, primers were also produce for site directed mutagenesis to produce a

A

Seprase Non-Catalytic Regions

```
            460                                                 500
BrCa        K YYALVCYGPG IPISTLHDGR TDQEIKILEE NKELENALKN
Melanoma    K YYALVCYGPG IPISTLHDGR TDQEIKILEE NKELENALKN
Chicken     K YYALICYGPG IPISTLFENE SDRELRILED NQELQSALQE

            501                                                 550
BrCa        IQLPKEEIKK LEVDEITLWY KMILPPQFDR SKKYPLLIQV YGGPCSQSVR
Melanoma    IQLPKEEIKK LEVDEITLWY KMILPPQFDR SKKYPLLIQV YGGPCSQSVR
Chicken     IILPKEEINK LEVDGITLWY KMLIPPQFDR SKKYPLLIQV YGGPCSQNVK

            551                                                 600
BrCa        SVFAVNWISY LASKEGMVIA LVDGRGTAFQ GDKLLYAVYR KLGVYEVEDQ
Melanoma    SVFAVNWISY LASKEGMVIA LVDGRGTAFQ GDKLLYAVYR KLGVYEVEDQ
Chicken     HTFSISWITY LASKEGIIVA LVDGRGTAYQ GDKILHAVYR RLGVYEVEDQ
```

B

Seprase Catalytic Domains

```
            601                                                 650
BrCa        ITAVRKFIEM GFIDEKRIAI WGWSYGGYVS SLALASGTGL FKCGIAVAPV
Melanoma    ITAVRKFIEM GFIDEKRIAI WGWSYGGYVS SLALASGTGL FKCGIAVAPV
Chicken     ISAVKKFIEM GFIDEKRIAI WGWSYGGYVT SLALGSGSGV FKCGIAVAPV

            651                                                 700
BrCa        SSWEYYASVY TERFMGLPTK DDNLEHYKNS TVMARAEYFR NVDYLLIHGT
Melanoma    SSWEYYASVY TERFMGLPTK DDNLEHYKNS TVMARAEYFR NVDYLLIHGT
Chicken     SSWEYYASIY TERFMGLPVE SDNLEHYKNS TVMARAKNFQ NVEYLLIHGT

            701 705
BrCa        ADDNV
Melanoma    ADDNV
Chicken     ADDNV
```

Figure 3. Deduced amino acid sequence alignments. Alignment of the deduced amino acid sequences for the partial chicken embryo seprase (Chicken), seprase from breast cancer cells (BrCa), and from human melanoma cells (Melanoma). Chicken seprase amino acids in purple are different from the human sequences. Amino acid sequence numbers for breast cancer seprase and human melanoma cell are identical to each other and shown above chicken embryo seprase.

(A) Alignment of deduced amino acid sequences of seprase from human breast cancer cells (BrCa), human melanoma cells (Melanoma) and chicken embryo (Chicken) in the non-catalytic region immediately adjacent to the catalytic domain.

(B) Alignment of deduced amino acid sequences of seprase from human breast cancer cells (BrCa), human melanoma cells (Melanoma) and chicken embryo (Chicken) in the catalytic domain. The conserved amino acids in red form the catalytic triad.

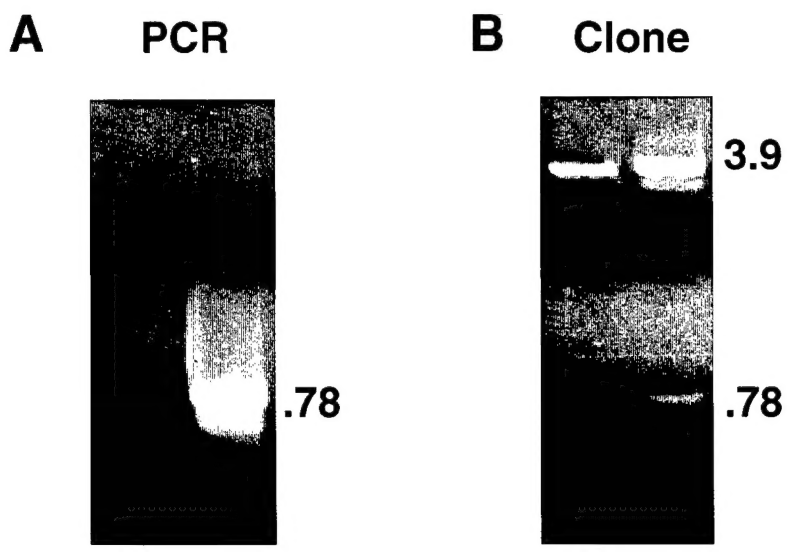

Figure 4. Cloning of the 0.78 kb chicken embryo seprase cDNA into the pcal-n expression vector. (A) PCR amplification of the 780 bp chicken embryo seprase cDNA to introduce restriction sites and a stop codon. Ethidium bromide stained agarose gel showing products from negative control PCR reaction without primers added (left lane) and 0.78 kb cDNA amplified by seprase specific primers (right lane). These two primers introduced a Bam HI and a Hind III restriction site on either end of the seprase cDNA so that the insert could be cloned into the vector in the correct frame and orientation for translation. In addition, a stop codon was introduced so that vector sequences wouldn't be translated.

(B) Restriction digestion of the fusion protein expression vector pcal-n to determine ligation of 0.78 kb cDNA insert. The pcal-n empty vector only (left lane) and vector with insert (right lane) were digested Bam HI and Hind III. The vector (3.9 kb) was detected in both cases (left & right lanes) but the 0.78 kb insert was only detected in the vector with insert (right lane).

wild type seprase cDNA as described below. These primers were:

Antisense: 5'-ACA GTG AAT CCT TGT GTT ACT CTG AA-3'
Sense: 5'-TTC AGA GTA ACA CAG GAT TCA CTG T-3'

In addition we have used the seprase cDNA inserts from the clones to produce ^{32}P-labeled seprase specific probes by random primer labeling. These probes have been used for northern blot analysis of seprase expression from human breast cancer cell lines (see Fig. 11, below).

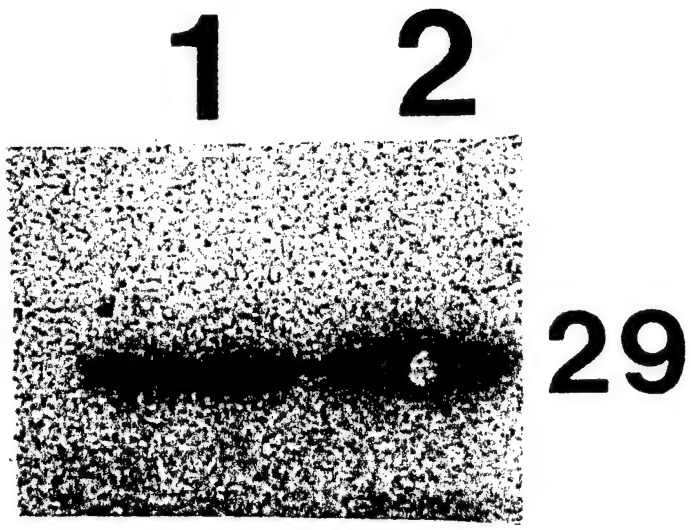

Figure 5. Expression of the calmodulin binding protein (CBP)-chicken embryo seprase fusion protein. This western blot reveals 29 kDa calmodulin-binding proteins in extracts of lysed IPTG-treated bacteria (lanes 1 & 2). The 29 kDa molecular weight corresponds to the predicted size of the CBP-chicken embryo seprase fusion protein. The original bacterial extract yielded insoluble and soluble fractions. The insoluble fraction was solubilized by re-extraction with detergent (lane 1) and loaded onto a SDS-PAGE with the original soluble fraction (lane 2). The nitrocellulose replicas were probed with biotinylated calmodulin and then streptavidin alkaline phosphatase. Calmodulin binding proteins were detected using the NBT-BCIP color substrate. Molecular weight is given x 10^{-3}.

II) Production of monoclonal antibodies directed against seprase.

Failure of anti-peptide antisera approach: Last year we presented ELISA results indicating that we had obtained antisera in mice that reacted with seprase specific peptides. Eventually, all mice injected with seprase-specific peptides produced sera with high titers versus the seprase specific peptides. Unfortunately, none of these sera was capable of recognizing native or denatured seprase as judged by Western blot or immunoprecipitation (not shown). This is a well known problem associated with making antisera to antigen-specific peptides. Therefore, this approach to develop new antibody probes to seprase was abandoned in favor of one that utilized the partial cDNA to chicken embryo seprase to produce a fusion protein that could be isolated and used for making antigen.

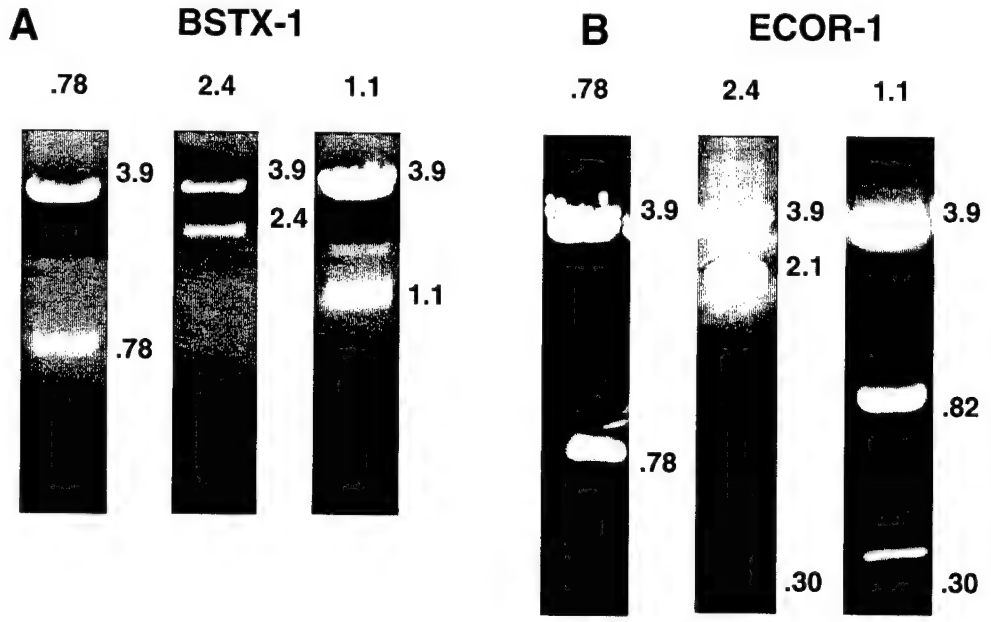

Figure 6. Restriction analysis of seprase cDNA clones. Ethidium bromide stained agarose gel revealing 3.9 kb and 0.78 kb, 2.4 kb and 1.1 kb restriction fragments generated by digestion of the pCR2.1 vector from plasmids having the 0.78 kb chicken embryo seprase cDNA (lane .78), 2.4 human breast cancer seprase cDNA (lane 2.4) and the 1.1 kb human breast cancer cDNA (lane 1.1) with BSTX-1 (A) and ECOR-1 (B). These fragments correspond to the predicted sizes of the plasmid (3.9 kb) and the partial chicken embryo seprase cDNA (0.78 kb), 2.4 kb human seprase and 1.1 kb human seprase. Note that the ECOR-1 digests of the 2.4 kb and 1.1 kb inserts reveals release of a 0.3 kb DNA (B, lanes 2.4 & 1.1).

Production of seprase catalytic domain-calmodulin binding protein (CBP) fusion protein to be used as antigen for generating antibodies directed against seprase. Complete characterization of the 780 bp partial cDNA for chicken embryos seprase was undertaken as the first step towards expressing a seprase fusion protein that could be used as immunogen to raise antibodies to seprase. Restriction analysis of pCR2.1 plasmid DNA isolated from bacterial clone #7 revealed that this clone harbored the correctly sized 780 bp insert (Figure 1). This plasmid was purified and sequenced by Model 377 automated DNA sequencing system in the Department of Microbiology & Immunology (Figure 2). This partial chicken embryo seprase sequence has 85 % homology to the published human melanoma sequence as judged by multiple sequence alignments using the GCG software available in the Department of Biochemistry & Molecular Biology. Comparison of the deduced amino acid sequence for chicken embryo seprase with those of human melanoma and of the human breast cancer sequences identified by our group (reported below) revealed high conservation of amino acids within the catalytic domain and less conservation of amino acids in the non-catalytic regions of the protein (Figure 3A & B).

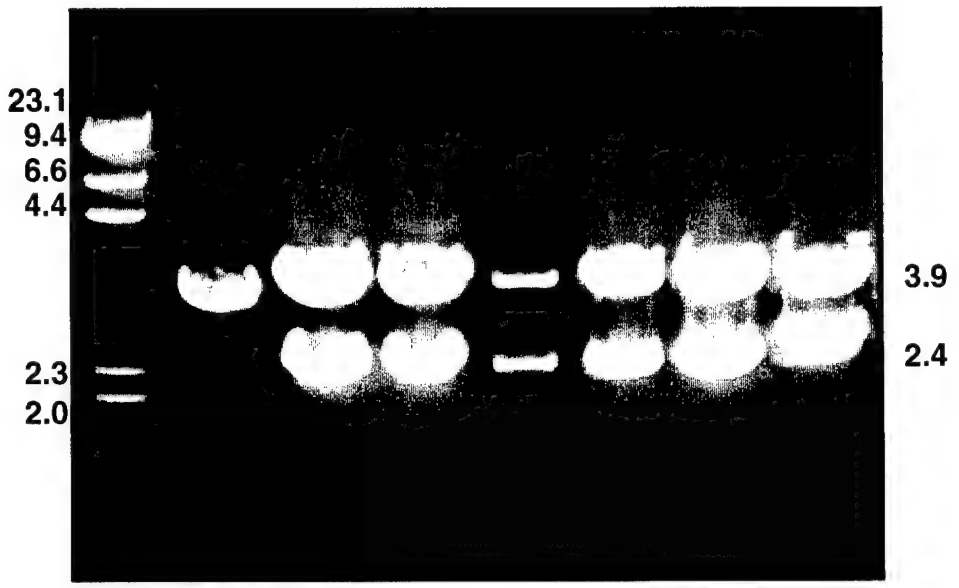

Figure 7. Restriction analysis revealing six clones with the 2.4 kb insert.
Ethidium bromide stained agarose gel revealing BSTX-1 restriction of the plasmids isolated from bacterial clones that grew up on from cells transformed with the 2.4 kb seprase cDNA (lanes 2-8). An empty vector identified in lane 2 that has only the 3.9 kb vector fragment. Lanes 3-8 reveal presence of a 2.4 kb insert DNA in addition to the 3.9 kb vector DNA. The plasmids were isolated from the following clones: lane 2, #55; lane 3, #48; lane 4, #49; lane 5, #50; lane 6, #51; lane 7, #56; lane 8, #58. Molecular sizes of the λ Hind III digest fragments (lane 1) are given on the left x 10^{-3}.

Expression of a chicken seprase-CBP fusion protein. The 780 bp cDNA was amplified by a PCR reaction with primers designed to introduce unique restriction sites on either end of the cDNA (Bam HI & Hind III) so that the insert could be cloned into the expression vector pcal-n (Stratagene, La Jolla, CA) in the correct frame and orientation for translation. In addition, a stop codon was introduced so that the vector sequence wouldn't be translated. The sense primer sequence was 5'-TTT GGA TCC GAA AGG TGC CAG TAT-3' and the antisense primer sequence was 5'-TTT AAG CTT ACA CAT TAT CAT CTG C-3'. PCR using this primer yielded the 780 bp amplicon as predicted (Figure 4A). The pcal-n expression vector and the PCR product were digested with Bam HI and Hind III and ligated together using the T4 DNA ligase. After ligation, the pcal-n was used to transform XL1-Blue competent cells and these were selected based on ampicillin resistance. Restriction of the fusion protein expression vector pcal-n with the 780 bp insert by BamHI and HindIII revealed that the 780 bp was included in the clones that grew up (Fig. 4B).

Seprase Catalytic Domain

```
            590                                                  639
BrCa56      RKLGVYEVED QITAVRKFIE MGFIDEKRIA IWGWSYGGYV SSLALASGTG
BrCa48      RKLGVYEVED QITAVRKFIE MGFIDEKRIA IWGWSYGGYV SSLALASGTG
BrCa49      RKLGVYEVED QITAVRKFIE MGFIDEKRIA IWGWSYGGYV SSLALASGTG
Melanoma    RKLGVYEVED QITAVRKFIE MGFIDEKRIA IWGWSYGGYV SSLALASGTG
            640                                                  689
BrCa56      LFKCGIAVAP VSSWEYYASV YTERFMGLPT KDDNLEHYKN STVMARAEYF
BrCa48      LFKCGIAVAP VSSWEYYASV YTERFMGLPT KDDNLEHYKN STVMARAEYF
BrCa49      LFKCGIAVAP VSSWEYYASV YTERFMGLPT KDDNLEHYKN STVMARAEYF
Melanoma    LFKCGIAVAP VSSWEYYASV YTERFMGLPT KDDNLEHYKN STVMARAEYF
            690                                                  739
BrCa56      RNVDYLLIHG TADDNVHFQN SAQIAKALVN AQVDFQAMWY SDQNHGLSGL
BrCa48      RNVDYLLIHG TADDNVHFQN SAQIAKALVN AQVDFQAMWY PDQNHGLSGL
BrCa49      RNVDYLLIHG TADDNVHFQN SAQIAKALVN AQVDFQAMWY SDQNHGLSGL
Melanoma    RNVDYLLIHG TADDNVHFQN SAQIAKALVN AQVDFQAMWY SDQNHGLSGL
```

Figure 8. Seprase catalytic domains of human breast cancer seprase. Alignment of deduced amino acid sequences of seprase from human breast cancer cells (BrCa56, BrCa48, BrCa49) and human melanoma cells (Melanoma) in the catalytic domain. The amino acids in red indicate the sequences that are absolutely conserved among all three sources and required to form the catalytic triad. There is complete conservation of all amino acids.

To express the fusion protein, several single XL1-Blue colonies were grown up and the plasmid vectors were purified and transformed into BL21 pLysS competent cells. After inducing insert expression with IPTG, the bacteria were lysed and separated into insoluble and soluble fractions. The insoluble fraction was then solublized by re-

extraction with detergent. Both fractions were analyzed by western blot with the antibody to the CBP-fusion protein. A 29-kDa protein band could be seen on the blot membrane for both fractions (Fig. 5). This protein corresponds to the predicted molecular size of the chicken embryo seprase-CBP fusion protein and it will be used for future antibody production.

F19 monoclonal antibody to FAP-α The sequence analysis of seprase-specific clones reveals that this protein is identical to the previously cloned fibroblast activation protein-alpha (FAP-α). Thus we have used this publicly available monoclonal antibody to FAP-α (F19) to investigate seprase expression in human breast cancer cell lines (see below: Figure 6).

SOW Task 3. Months 3-18: Identify clones with full-length seprase cDNA inserts.

Seprase Sequences

```
           588                                                  637
CeSep      VYRRLGVYEV EDQISAVKKF IEMGFIDEKR IAIWGWSYGG YVTSLALGSG
BrCa1.1    VYRKLGVYEV EDQITAVRKF IEMGFIDEKR IAIWGWSYGG YVSSLALASG
BrCa2.4    VYRKLGVYEV EDQITAVRKF IEMGFIDEKR IAIWGWSYGG YVSSLALASG
Melanoma   VYRKLGVYEV EDQITAVRKF IEMGFIDEKR IAIWGWSYGG YVSSLALASG
FAP-a      VYRKLGVYEV EDQITAVRKF IEMGFIDEKR IAIWGWSYGG YVSSLALASG

           638                                                  687
CeSep      SGVFKCGIAV APVSSWEYYA SIYTERFMGL PVESDNLEHY KNSTVMARAK
BrCa1.1    TGLFKCGIAV APVSSWEYYA SVYTERFMGL PTKDDNLEHY KNSTVMARAE
BrCa2.4    TGLFKCGIAV APVSSWEYYA SVYTERFMGL PTKDDNLEHY KNSTVMARAE
Melanoma   TGLFKCGIAV APVSSWEYYA SVYTERFMGL PTKDDNLEHY KNSTVMARAE
FAP-a      TGLFKCGIAV APVSSWEYYA SVYTERFMGL PTKDDNLEHY KNSTVMARAE

           688                                                  737
CeSep      NFQNVEYLLI HGTADDNVK
BrCa1.1    YFRNVDYLLI HGTADDNVHF QNSAQIAKAL VNAQVDFQAM WYSDQNHGLS
BrCa2.4    YFRNVDYLLI HGTADDNVHF QNSAQIAKAL VNAQVDFQAM WYSDQNHGLS
Melanoma   YFRNVDYLLI HGTADDNVHF QNSAQIAKAL VNAQVDFQAM WYSDQNHGLS
FAP-a      YFRNVDYLLI HGTADDNVHF QNSAQIAKAL VNAQVDFQAM WYSDQNHGLS
```

Figure 9. Comparison of catalytic domains of seprase from different sources. Alignment of deduced amino acid sequences of seprase from chicken embyros (CeSep), 1.1 kb amplicon from human breast cancer cells (BrCa1.1), 2.4 amplicon from human breast cancer cells (BrCa 2.4), human melanoma cells (Melanoma) and FAP-α in the catalytic domain. The amino acids in red indicate the sequences that are absolutely conserved among all three sources and required to form the catalytic triad. The seprase/FAP-α from human sources is exactly conserved. Divergent amino acids in the chicken sequence are indicated in purple.

Human breast cancer seprase: As reported last year, RT-PCR using RNA from MDA-MB-436 cells and seprase-specific primers FAP1 (5'-CCACGCTCTGAAGACAGAATT-3') and FAP 6 (5'-TCAGATTCTGATACAGGCT-3') led to amplification of two reaction products of 2.4 kb and 1.1 kb. These full length (2.4 kb) seprase cDNAs and 1.1 kb seprase cDNAs have been cloned into the pCR2.1 vector (Invitrogen) as judged by restriction analysis of plasmid DNA isolated from ampicillin resistant transformed bacteria (Figs. 6 & 7). Several clones of both 2.4 kb and 1.1 kb seprase have been plaque purified and stored as glycerol stocks. Thus this task is now successfully completed.

The 2.4 and 1.1 kb RT-PCR amplicons were isolated by gel purification and ligated into the pCR2.1 TOPO cloning vector (Invitrogen). For each cDNA, transformed bacteria were grown on 5 separate agar plates containing ampicillin and x-gal. After overnight culture the transformants were analysed by blue/white selection with white colonies indicating those harboring an insert and blue colonies likely to have empty vector. For the 2.4 kb insert there were 79 white colonies and 198 blue colonies for a total of 277

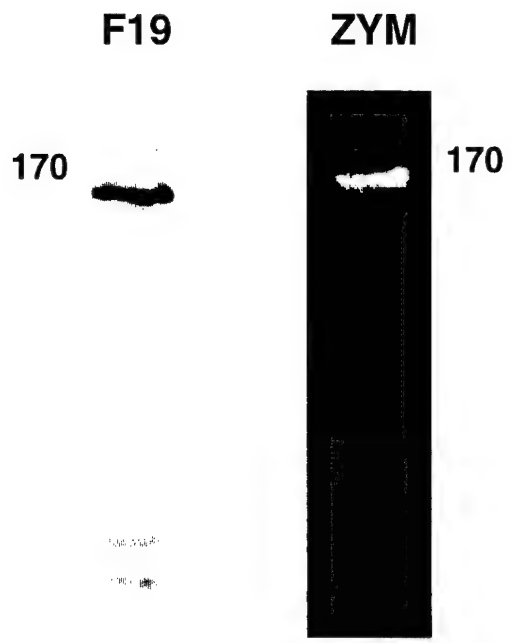

Figure 10. F19 mAb to FAP-α identifies seprase from MDA-MB-436 human breast cancer cells. This autoradiogram reveals seprase in detergent extracts of MDA-MB-436 human breast cancer cells detected by mAb F19 as a 170 kDa protein by Western blot (F19). Seprase in the MDA-MB-436 extracts is confirmed by its gelatinase activity that is visualized as an unstained 170 kDa band on this gelatin zymogram (ZYM). Molecular weights are given x 10^{-3}.

colonies. The 1.1 kb cloning yielded 83 white colonies and 57 blue colonies for a total of 140 colonies. Ten individual white colonies were picked for each cDNA and grown up in overnight culture. Plasmids were purified and analyzed by restriction with BSTX-1 an enzyme that cleaves on either side of the insert but should not restrict a seprase insert. This resulted in liberation of 2.4 kb and 1.1 kb inserts from there respective plasmids (Fig. 6A). Similar results were obtained with ECOR-1 (Fig. 6B). Interestingly, ECOR-1 cleaves on once on either side of the insert in the polylinker region; however, according to the sequence of melanoma seprase, there should also be a single internal ECOR-1 restriction site in the seprase open reading frame. Thus, restriction of 2.4 kb seprase with ECOR-1 should yield insert fragments of 2.1 and 0.3 kb while restriction of the 1.1 kb sequence should yield fragments of 0.8 and 0.3 kb. This is exactly the results obtained suggesting that seprase-specific cDNAs were cloned (Fig. 6B). Several cDNAs with the 2.4 kb insert were cloned (Fig. 7).

SOW Task 4 Months 4-24: Confirm full-length seprase cDNA clones.

The seprase 2.4 kb and 1.1 kb cDNAs have been cloned and sequenced. The

Seprase expression in human breast cell lines

Figure 11. Northern blot analysis of seprase expression by human breast cancer cell lines. Phosphorimage of a nitrocellulose replica of total RNA that was extracted from different human breast cancer cell lines and loaded onto the gel (20 μg/lane) from left to right as follows: 1) MDA-MB-231; 2) MDA-MB-436; 3) MDA-MB-435s; and 4) MCF-7.

Seprase expression: The top panel was probed with ^{32}P-random-primer labeled 2.4 kb seprase cDNA from clone 49 (34 x 10^6 cpm). A single band at approximately 5 kb is detected in the MDA-MB-436 and MDA-MB-435s RNA (middle two lanes). No seprase-specific message is detected in the MDA-MB-231 lane (first lane on the left) or the MCF-7 lane (last lane on the right).

β-**actin expression** Lower panel shows the phophorimage of the same nitrocellulose replica used for the seprase expression determination that was stripped and re-probed with ^{32}P-random primer labeled β-actin probe (42 x 10^6 cpm). High levels of β-actin message are detected in the total RNA from all four human breast cancer cell lines.

sequences have been compared to the seprase sequences published for human melanoma seprase (Genbank accession number U76833) and human FAP-α (Appendix, DNA sequence alignments). Thus this task is complete as documented below

Characterization of the full-length human breast cancer seprase cDNAs. Four 2.4 kb clones were sequenced (48, 49, 51, and 56) and shown to be very highly homologous to human breast cancer seprase and FAP-α. Part way through the sequence analysis clone 51 failed to grow, so characterization is only complete for clones 48, 49,and 56.

Assembly of the complete 2.4 kb seprase cDNA sequences Complete sequences

were assembled by combining consecutively overlapping sequence data from obtained from automated sequencing reactions with the following primers (primer sequences listed above): M13 forward primer; primer 2, primer 9, primer 7 (reverse), and primer 1. Sequences obtained using M13 reverse primer, primer 5 (reverse), and primer 6 were used to confirm sequences. For example, the complete sequence of clone 49 was assembled using sequences primed by: M13 forward (nucleotides 1-585); Primer 2 (nucleotides 586-1123); Primer 9 (nucleotides 1124-1578); Primer 7 (reversed, nucleotides 1579-1928) and Primer 1 (nucleotides 1929-2366). In general, each automated sequencing reaction yielded approximately 1100 nucleotides of sequence and typically, there were no ambiguous bases within the first 600 nucleotides. For assembly, the raw sequences were trimmed of all sequences occurring after the first ambiguous base. Then, the correct merging point was identified by pair-wise sequence alignment using the GCG sequence analysis programs. The assembled sequences were analyzed for open reading frames using the GCG sequence analysis software. Clones 48 and 49 have a single long open reading frame that includes the entire seprase sequence (see sequence alignment in appendix). However, clone 48 has a significant deletion of 45 nucleic acids between 1143 and 1191 relative to melanoma seprase and to the other breast cancer seprase clones. Clone 56 is closely homologous to clones 48 and 49 but has its open reading frame interrupted due to 7 additional bases inserted through out its sequence.

Comparison of the human breast cancer seprase clone 49 sequence with that of human melanoma seprase revealed three single base differences. At position 1418 clone 49 has a "G" while melanoma seprase has an "A". At position 2095 clone 49 has a "C" and melanoma seprase has a "T". At position 2416 clone 49 has a "G" while melanoma seprase has an "A" (Appendix, highlighted residues, multiple nucleic acid sequence alignment). Translation of the 49 sequence revealed an amino acid sequence identical to that of human melanoma seprase except that 49 has alanine at position 404 in the protein instead of the threonine found in human melanoma seprase (Appendix; Translation alignment). This amino acid change is due to the base difference at position 1418 and is likely an error introduced by the RT-PCR procedure because clones 48 and 56 have the correct nucleotide in this position (Appendix, multiple sequence alignments).

A site directed mutagenesis procedure was performed to produce a cDNA encoding "wild-type" human breast cancer seprase. To change the G at position 1418 to an A, oligonucleotide a sense primer was synthesized that were over 20 amino acids long and corresponded exactly to the sequences on either side of position 1418 but had inserted the A at position 1418 instead of the G (5'-TTC AGA GTA ACA CAG GAT TCA CTG T-3'). An antisense primer was also synthesized as above that is identical to the sequence except that it replaces the mutant C with a T at position 1418 (5'-ACA GTG AAT CCT TGT GTT ACT CTG AA-3'). The sense primer was used with the M13

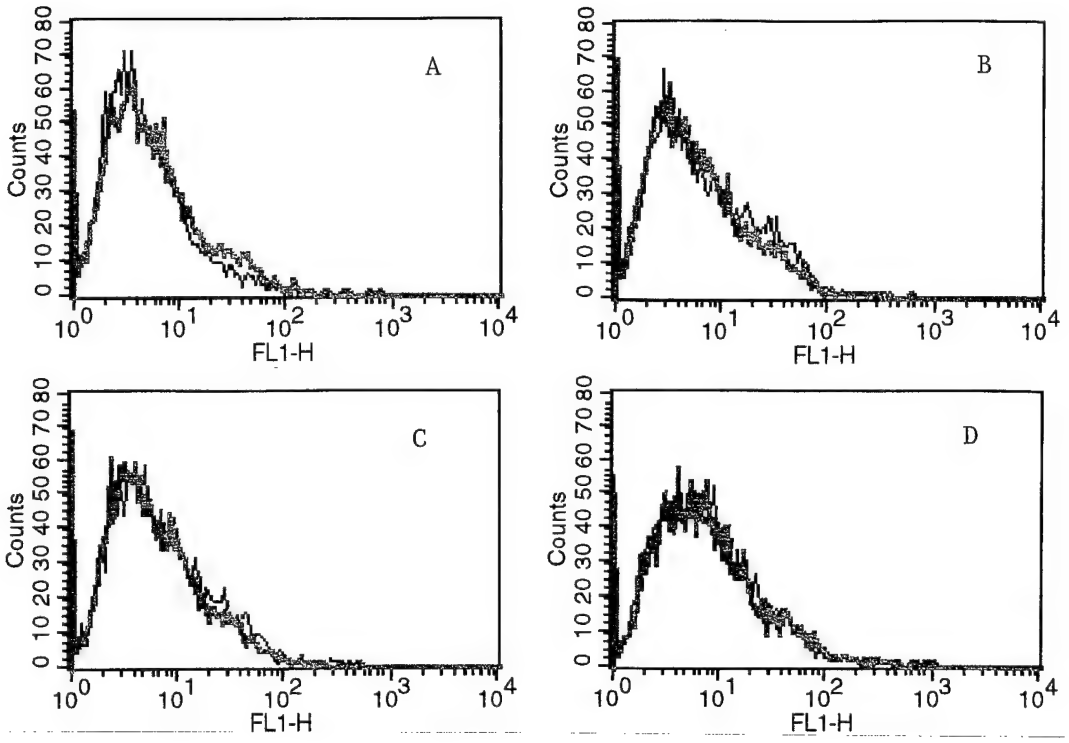

Figure 12. MCF-7 human breast cancer cell-transfectants with seprase in the sense orientation fail to express seprase on their surfaces. FACS analysis of MCF-7 human breast cancer cells stained with F19 (red traces) or without F19 (black traces). No increase in fluorescence intensity is detected in any of the transfectants and the unstained black lines are in the same position as the red lines for cells stained with F19 to detect seprase. The cells tested are: A) Untransfected MCF-7; B) MCF-7 transfected with seprase clone 48; C) MCF-7 transfected with seprase clone 49; and D) MCF-7 transfected with seprase clone 56.

reverse primer and clone 49 template for PCR with the pfu DNA polymerase to produce an approximately 1 kb cDNA specific from positions 1335 to the 2366 (relative to melanoma seprase numbering). The antisense primer was used in conjunction with the M13 forward primer to produce a 1.3 kb cDNA encoding positions 1-1355. The two PCR products were gel purified and then used as templates for PCR reaction using only the M13 forward and reverse primers to generate the complete 2.4 kb sequence. This procedure did produce the 2.4 kb product and this was successfully cloned into the pCR2.1 cloning vector (not shown). Sequence analysis on this cDNA is not complete. However, we anticipate that we have indeed produced the wild type full length human breast cancer seprase cDNA.

We conclude that we have cloned a full-length seprase cDNA from human breast cancer cells. This cDNA is virtually identical to that reported for human melanoma seprase and FAP-α. The sequence of clone 49 should encode for a fully functional protein; however, we have used site directed mutagenesis to produce an exact wild type seprase. Transfection experiments have been initiated with all three clones

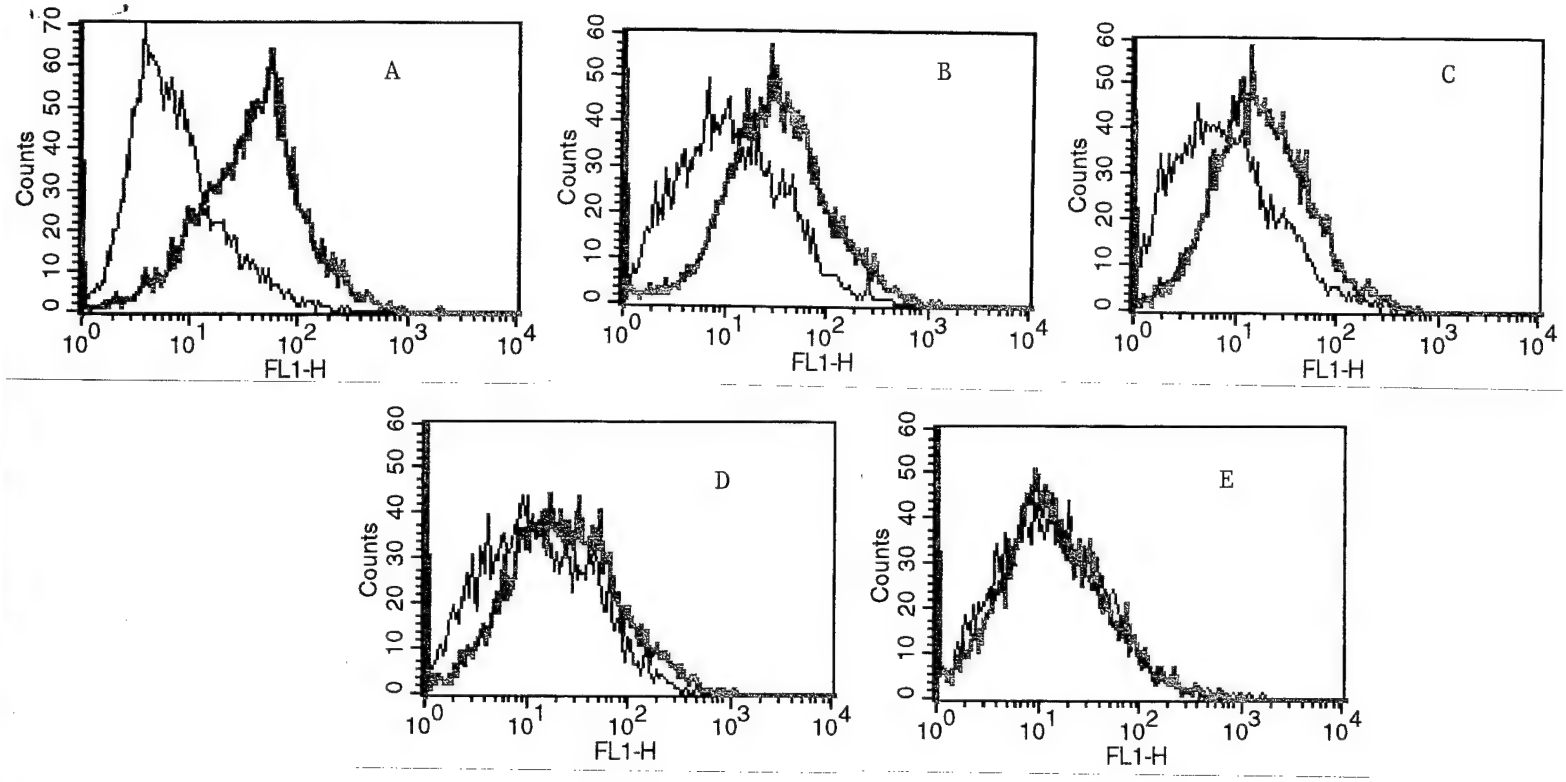

Figure 13. Down-regulation of seprase in MDA-MB-436 human breast cancer cells.
FACS analysis of different MDA-MB-436 human breast cancer cells stained with F19 (red traces) or without F19 (black traces). A) MDA-MB-436 cells stained with F19 antibody to seprase are more intensely stained than control. B) Control vector-only transfected cells also have high level of seprase staining. C) MDA-MB-436 cells transfected with antisense seprase clone 48 still have high surface expression of seprase. D) MDA-MB-436 cells transfected with antisense seprase clone 49 appear to have somewhat decreased surface expression of seprase that approaches control levels. E) MDA-MB-436 cells transfected with antisense seprase clone 56 seprase expression levels are indistinguishable from the unstained control.

(described below) and will begin for the wild type clone upon confirmation of its sequence.

1.1 kb clones are seprase sequences that apparently are inappropriately amplified products of the RT-PCR. Four 1.1 kb clones (70, 71, 78 and 80) been completely sequenced. Although these clones bear high sequence homology to seprase and have long open reading frames, none appear to code for a single open reading frame after a start codon. Moreover, Northern blot analysis has only identified a single message at approximately 5 kb (see below). Thus we currently believe that the 1.1 kb cDNA amplicon is a spurious product of the RT-PCR procedure and not a naturally occurring alternatively spliced product. We will focus effort on the full-length seprase clones.

SOW Task 5 Months 18-36: Produce stable transfectants of malignant and normal breast cells that overexpress assembled, active seprase to the cell surface. Task 5

months 18-36

The DNA sequence information has revealed that human breast cancer cells produce the same seprase as produced by human melanoma cells. Moreover, seprase cloned from these two sources is identical to FAP-α cloned from reactive human fibroblasts. Thus, we investigated the possibility that a FAP-α antibody could identify seprase produced by human breast cancer cells. Seprase was identified by monoclonal antibody F19 to FAP-α in extracts of MDA-MB-436 breast cancer cells (Fig. 10, F19). Moreover, seprase activity was also identified in these cells (Fig. 10, ZYM) indicating that they produce a functional and fully assembled seprase. Thus, the F19 antibody reacts with seprase and has been used to monitor the cell surface expression of seprase by FACS analysis (described below).

Northern blot analysis reveals that the MDA-MB-436 and the MDA-MB-435s invasive breast cancer cell lines express high levels of seprase but seprase is not detected in the non-invasive breast cancer cell line MCF-7 (Fig. 11). Seprase mRNA was also not detected in the invasive human breast cancer cell line MDA-MB-231 (Fig. 11). The fact that seprase is over-expressed by human breast cancer cells is somewhat controversial given that FAP-α is reportedly only expressed by reactive stromal fibroblasts in epithelial tumors, including breast cancer (4). We have reported that seprase, which we now know is identical to FAP-α, is over-expressed by malignant cells of human breast cancers (15). A manuscript is now in preparation reporting direct evidence for seprase over-expression in human breast cancer cell lines based on our data on sequencing and expression of human breast cancer seprase (see abstract of a manuscript in preparation in the reportable outcomes section).

Two sets of transfection experiments were simultaneously begun. We began trying to produce stable transfectants of MCF-7 cells that over-express functional seprase as well as stable transfectants of MDA-MB-436 cells that have seprase specifically down-regulated.

Transfections of cells MCF-7 human breast cancer cells with seprase in the sense orientation.

MCF-7 human breast cancer cells do not express detectable levels of seprase and are not invasive (Fig. 11). These cells are being used to produce transfectants that produce assembled proteolytically active seprase to the cell surface. To get the seprase cDNA into a mammalian expression vector it was excised from the pCR2.1 cloning vector by restriction with Bam H1 and Apa1. The seprase DNA was separated from the vector DNA by agarose gel electrophoresis, purified from the gel and ligated in the sense orientation into Bam H1 and Apa1 restricted pcDNA3.1 mammalian expression vector (Promega). MCF-7 cells at 75 % confluence were transformed with 2

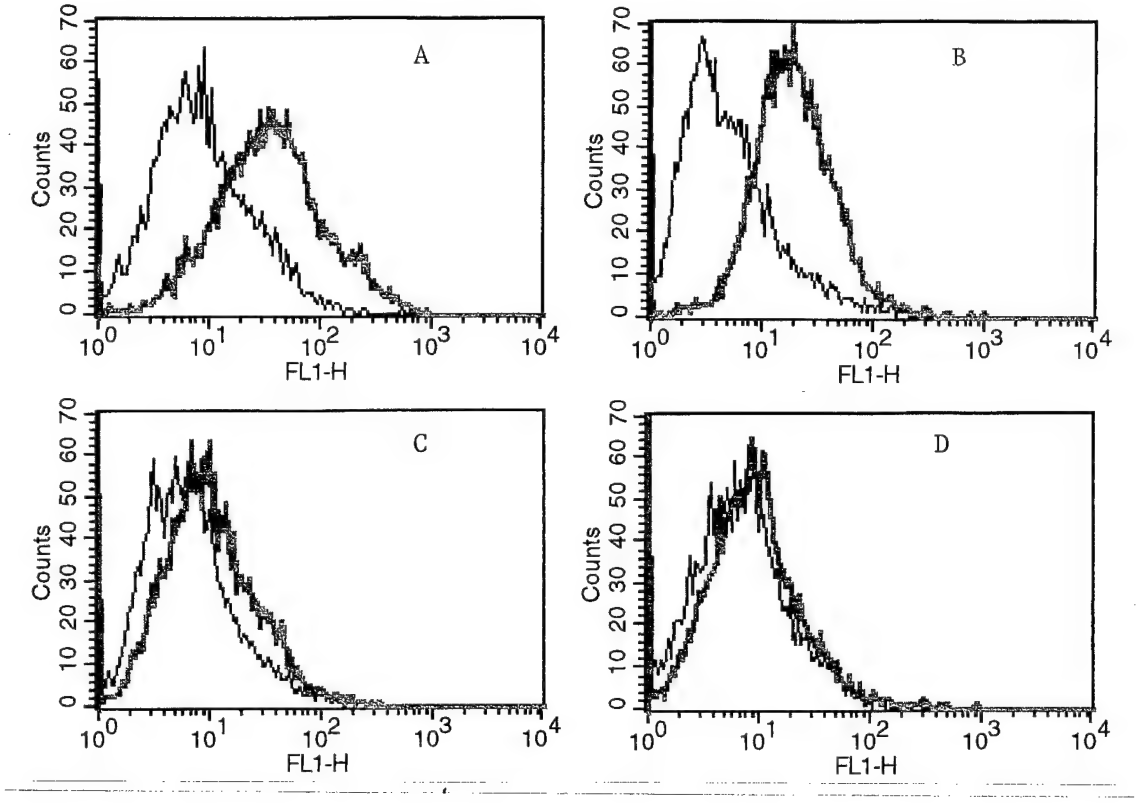

Figure 14. Down-regulation of seprase in clonal transfectants of MDA-MB-436 human breast cancer cells.

FACS analysis of different MDA-MB-436 human breast cancer cells stained with F19 (red traces) or without F19 (black traces). A) Vector-only transfectant clone E8 of MDA-MB-436 cells stained with F19 antibody to seprase reveals a high level of seprase expression. B) Antisense seprase transfectant clone 48E9 also has a high level of seprase staining. C) Antisense seprase transfectant clone 56E10 has decreased seprase expression. D) Antisense seprase transfectant 56H5 has greatly decreased surface expression of seprase that approaches control levels.

μg pcDNA3.1 having insert DNA from clones 48, 49 or 56 using lipofectin (GIBCO-BRL). Transfectants were selected by growth in medium containing G418 (400 μg/mL). When the transfectants had grown up, they were harvested by treating with EDTA and analyzed by FACS using F19 as the primary antibody and FITC-labeled goat anti-mouse IgG (Zymed) (Fig. 12 red traces). Background fluorescence was determined by leaving out the primary antibody and staining the cells with FITC-labeled goat anti-mouse IgG (Fig. 12 black traces). Unfortunately, FACS analysis of all three transfectants on two separate occasions failed to detect any increase in cell-surface seprase (Fig. 12 A-D). Thus, we are concerned that the mutations in these clones are preventing proper production and expression to the cell surface of the seprase. Our plan is to use the "wild-type" clone 49 for transfecting these cells as soon as the sequence is confirmed.

Down-regulation of cell-surface seprase by transfections of MDA-MB-436 with seprase cDNAs in the antisense orientation.

Full length seprase cDNAs included in clones 48, 49, and 56 were also ligated into the pcDNA3.1 - vector by the strategy outlined above. This vector causes expression of antisense seprase mRNA. MDA-MB-436 cells were transfected with anti-sense seprase from clone 48, 49 or 56 as described above. FACS analysis with F19 was performed on the cells that grew up following selection with G418. The results revealed that wild type MDA-MB-436 cells, control transfectants lacking the seprase insert and cells transfected with antisense clone 48 maintained high levels of seprase expression (Fig. 13 A-C), while those transfected with antisense clone 49 and clone 56 had decreased expression of seprase (Fig. 13 D & E). The cells transfected with 49 had reduced but not complete loss of seprase expression (Fig. 13 D). However, the cells transfected with 56 had lost seprase expression down to back ground levels of cells stained with only the FITC-goat anti-mouse IgG and not stained with the F19 antibody to seprase (Fig. 13 E). This very exciting finding prompted us to clone the transfectants for 49 and 56 by limiting dilution.

A clone that grew from the control transfectants and clones that grew up from the cells transfected with 49 did not exhibit a down-regulation of seprase expression as judged by FACS with F19 (Fig. 14 A & B). However, the two clones that grew up from the cells transfected with 56 had decreased seprase expression (Fig. 14 C & D). These results were repeated after growing the cells for a few more weeks (Antisense clones 2) confirming that the phenotype is stable with respect to down-regulation of seprase. Based on these results, nude mice were ordered to determine the invasive phenotypes of seprase over-expressing cells (wild type and cloned control transfected MDA-MB-436 cells) as compared to the invasive phenotypes of cells down-regulated for seprase expression (56H5 and 56E10 seprase antisense clonal transfectants of MDA-MB-436).

Task 6. Months 24-48: Determine effects of seprase overexpression on cell-mediated matrix proteolysis.

Preliminary results comparing the wild type and control-transfected MDA-MB-436 cells to their seprase down-regulated counterparts suggests that seprase may have a role in stimulating cell growth. The 56H5 clonal transfectant of seprase has undetectable surface levels of seprase and grows very slowly relative to all other MDA-MB-436 cells (Fig. 13 D). More clones are needed to determine if this is a peculiarity of this clone that is unrelated to seprase expression or if it is correlated with loss of seprase. Interestingly, the 56E10 antisense transfectant apparently grows normally. However, this transfectant has reduced but detectable levels of seprase on its surface (Fig. 13 C). Thus, experiments to measure possible effects of loss of seprase on breast cancer cell proliferation are being pursued.

The antisense transfectants with reduced seprase levels now enables us to pursue the

proposed in vitro extracellular matrix proteolysis experiments. This task is now begun and should be completed by the end of the project.

Task 7. Months 30-48: Determine effects of seprase overexpression on breast cell invasion of extracellular matrix.

Using the antisense seprase transfectants, we have begun an animal experiment to determine the role of seprase in breast cancer cell invasion. Four groups of seven Balb c nu/nu mice have been purchased and given 60 day estrogen release pellets as described in the original proposal. Each mouse has been injected ID into the mammary fat pads at 4 different sites with 2 x 10^6 cells per site. The groups are mice injected with: 1) MDA-MB-436 wild type cells; 2) MDA-MB-436 3.1E8 control transfectants; 3) partial seprase down-regulated MDA-MB-436 56E10 seprase antisense transfectants and 4) total seprase down-regulated MDA-MB-436 56H5 antisense transfectants. After 11 days the tumor take has been very good with groups 1,2 & 3 having X tumors per 28 injection sites. However, after 12 days the animals in group 4 have only a 50 % tumor take, consistent with the notion that these cells do not grow as well as wild type and control-transfected MDA-MB-436 cells. When the tumors become large but before the animals show signs of distress, the animals will be sacrificed. Detailed analysis of tumor invasion and growth will be conducted.

Key Research Accomplishments this year:

- Characterization of a partial cDNA for chicken embryo seprase
- Production of a fusion protein of chicken embryo seprase-calmodulin binding protein.
- Molecular cloning of a full-length 2.4 kb seprase cDNA from human breast cancer cells.
- Molecular cloning of a 1.1 kb seprase cDNA from human breast cancer cells
- Complete nucleic acid sequence analysis of the seprase cDNAs.
- Direct demonstration of seprase over-expression by Northern blot analysis of human breast cancer cell lines.
- Demonstration of functional seprase protein naturally occurs in breast cancer cell lines by immunoblot and zymographic analyses.
- Successful down-regulation of seprase by transfection of human breast cancer cells with seprase cDNA expressed in the antisense orientation.
- Initiation of animal studies of breast cancer cell growth and invasion using wild type, control transfectants, and transfectants of human breast cancer cells with decreased seprase expression.

Reportable Outcomes:

Papers out since last report: Included in the appendix

Kelly, T., Y. Yan, R.L. Osborne, A.B. Athota, T.L. Rozypal, J.C. Colclasure, and W.S. Chu (1998). Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases. *Clin. Exp. Metastasis.* 16:501-512.

Kelly, T. (1999). Evaluation of seprase activity. *Clin. Exp. Metastasis. 17*:67-72.

Abstract of manuscript in preparation:

Overexpression of seprase by human breast cancer cell lines. Tricia L. Rozypal and Thomas Kelly, Department of Pathology, Arkansas Cancer Research Center, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199

Seprase is a cell surface, serine protease that degrades extracellular matrix and may facilitate tumor invasion. Molecular cloning reveals that seprase is identical to fibroblast activation protein-α (FAP-α) and immunohistochemical studies show that seprase/FAP-α is overexpressed in tissue specimens of human breast cancer. However, reports conflict regarding the types of cells that express seprase/FAP-α in these tumors. Specifically, the FAP-α monoclonal antibody (F19) labels reactive stromal fibroblasts and does not label the malignant cells while a seprase antiserum reacts most strongly with breast carcinoma cells (Scanlan et al, 1994, Proc. Natl. Acad. Sci. USA, 91:5657-61; Kelly et al, 1998, Mod. Pathol., 11:855-63). Here we investigated seprase expression in several human breast cell lines. A full length, 2.4 kb seprase/FAP-α cDNA was amplified by RT-PCR and cloned from the MDA-MB-436 human breast cancer cell line. This cDNA comprises a single open reading frame spanning the entire coding region of seprase/FAP-α and having 99.8 % nucleic acid sequence identity to seprase/FAP-α. Zymographic, immunoblot and northern blot analyses reveal that a low level of seprase is expressed by the non-invasive HBL-100 and MCF-7 human breast cell lines. Seprase/FAP-α is not detected in extracts of invasive MDA-MB-231 human breast cancer cells; however, it is expressed to high levels by two other invasive human breast cancer cell lines (MDA-MB-435s and MDA-MB-436). These results directly demonstrate that human breast cancer cells can over-express seprase/FAP-α. Supported by DoD, Department of the Army, grant DAMD17-96-1-6097.

Conclusions:

This past year has been successful and much progress has been made towards completing the goals of the project. Moreover, we are on schedule to complete the project on time. Specifically, we have cloned full-length seprase cDNAs from the MDA-MB-436 human breast cancer cell line. DNA sequence analysis has confirmed the clones and enabled the production of seprase specific cDNA probes. Seprase specific probes directly demonstrated that seprase is over-expressed by invasive human breast cancer cells. The sequence analysis also reveals that seprase from human breast cancer is identical to that expressed by human melanoma cells and also to the previously cloned FAP-α. The fact that seprase is expressed by wild type MDA-MB-436 cells has been demonstrated by immunoblot analysis with F19 monoclonal antibody to seprase/ FAP-α, by identification of seprase activity using gelatin zymography and by F19 surface labeling of MDA-MB-436 cells as detected by FACS. Investigation into the role of seprase in breast cancer invasion and metastasis has begun both in vivo using the nude mouse model and in vitro. Two papers came out over this past year as a direct result of this research and there is one manuscript in preparation. Thus, this research continues to be productive, providing new information about seprase and breast cancer cell invasion.

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Appendix

Role of seprase in breast cancer invasion
Third year progress report
October 7, 1999

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Little Rock, AR 72205-7199

Includes: Multiple DNA sequence alignments of clones 48,49, & 56 with seprase and FAP-α

Amino acid alignment of clone 48 & seprase

2 Papers

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Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   56f_1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
     49f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
     48f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          51                                                   100
Hsu09278  TGGCACGGTA TTCAAAAGTC CGTGGAAAGA AAAAAACCTT GTCCTGGCTT
Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   56f_1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~ACGAC TCACTATAGG GCGAATTGGG
     49f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~ATACGAC TCACTATAGG GCGAATTGGG
     48f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~CGAC TCACTATAGG GCGAATTGGG

          101                                                  150
Hsu09278  CAGCTTCCAA CTACAAAGAC AGACTTGGTC CTTTTCAACG GTTTTCACAG
Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   56f_1  CCCTCTAGAT GCATGCTCGA GCGGCCGCCA GTGTGATGGA TATCTGCAGA
     49f  CCCTCTAGAT GCATGCTCGA GCGGCCGCCA GTGTGATGGA TATCTGCAGA
     48f  CCCTCTAGAT GCATGCTCGA GCGGCCGCCA GTGTGATGGA TATCTGCAGA

          151                                                  200
Hsu09278  ATCCAGTGAC CCACGCTCTG AAGACAGAAT TAGCTAACTT TCAAAAACAT
Hsu76833  ~~~~~~~~~~ CCACGCTCTG AAGACAGAAT TAGCTAACTT TCAAAAACAT
   56f_1  ATTCGCCCTT CCACGCTCTC AAGACAGAAT TAGCTAACTT TCAAAAACAT
     49f  ATTCGCCCTT CCACGCTCTC AAGACAGAAT TAGCTAACTT TCAAAAACAT
     48f  ATTCGCCCTT CCACGCTCTC AAGACAGAAT TAGCTAACTT TCAAAAACAT

          201                                                  250
Hsu09278  CTGGAAAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT TGCCACCTCT
Hsu76833  CTGGAAAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT TGCCACCTCT
   56f_1  CTGGAAAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT TGCCACCTCT
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     49f  CTGGAAAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT TGCCACCTCT
     48f  CTGGAAAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT TGCCACCTCT

          251                                                  300
Hsu09278  GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT
Hsu76833  GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT
  56f_1   GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT
     49f  GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT
     48f  GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT

          301                                                  350
Hsu09278  TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCACACTG AAGGATATTT
Hsu76833  TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCACACTG AAGGATATTT
  56f_1   TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCACACTG AAGGATATTT
     49f  TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCACACTG AAGGATATTT
     48f  TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCACACTG AAGGATATTT

          351                                                  400
Hsu09278  TAAATGGAAC ATTTTCTTAT AAAACATTTT TTCCAAACTG GATTTCAGGA
Hsu76833  TAAATGGAAC ATTTTCTTAT AAAACATTTT TTCCAAACTG GATTTCAGGA
  56f_1   TAAATGGAAC ATTTTCTTAT AAAACATTTT TTCCAAACTG GATTTCAGGA
     49f  TAAATGGAAC ATTTTCTTAT AAAACATTTT TTCCAAACTG GATTTCAGGA
     48f  TAAATGGAAC ATTTTCTTAT AAAACATTTT TTCCAAACTG GATTTCAGGA

          401                                                  450
Hsu09278  CAAGAATATC TTCATCAATC TGCAGATAAC AATATAGTAC TTTATAATAT
Hsu76833  CAAGAATATC TTCATCAATC TGCAGATAAC AATATAGTAC TTTATAATAT
  56f_1   CAAGAATATC TTCATCAATC TGCAGATAAC AATATAGTAC TTTATAATAT
     49f  CAAGAATATC TTCATCAATC TGCAGATAAC AATATAGTAC TTTATAATAT
     48f  CACGAATATC TTCATCAATC TGCAGATAAC AATATAGTAC TTTATAATAT

          451                                                  500
Hsu09278  TGAAACAGGA CAATCATATA CCATTTTGAG TAATAGAACC ATGAAAAGTG
Hsu76833  TGAAACAGGG CAATCATATA CCATTTTGAG TAATAGAACC ATGAAAAGTG
  56f_1   TGAAACAGGA CAATCATATA CCATTTTGAG TAATAGAACC ATGAAAAGTG
     49f  TGAAACAGGA CAATCATATA CCATTTTGAG TAATAGAACC ATGAAAAGTG
     48f  TGAAACAGGA CAATCATATA CCATTTTGAG TAATAGAACC ATGAAAAGTG

          501                                                  550
Hsu09278  TGAATGCTTC AAATTACGGC TTATCACCTG ATCGGCAATT TGTATATCTA
Hsu76833  TGAATGCTTC AAATTACGGC TTATCACCTG ATCGGCAATT TGTATATCTA
  56f_1   TGAATGCCTC AAATTACGGC TTATCACCTG ATCGGCAATT TGTATATCTA
     49f  TGAATGCTTC AAATTACGGC TTATCACCTG ATCGGCAATT TGTATATCTA
     48f  TGAATGCTTC AAATTACGGC TTATCACCTG ATCGGCAATT TGTATATCTA

          551                                                  600
Hsu09278  GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACACAG CAACATATTA
Hsu76833  GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACACAG CAACATATTA
  56f_1   GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACACAG CAACATATTA
     49f  GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACACAG CAACATATTA
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     48f  GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACACAG CAACATATTA

          601                                                  650
Hsu09278  CATCTATGAC CTTAGCAATG GAGAATTTGT AAGAGGAAAT GAGCTTCCTC
Hsu76833  CATCTATGAC CTTAGCAATG GAGAATTTGT AAGAGGAAAT GAGCTTCCTC
   56f_1  CATCTATGAC CTTAGCAATG GAGAATTTGT AAGAGGAAAT GAGCTTCCTC
     49f  CATCTATGAC CTTAGCAATG GAGAATTTGT AAGAGGAAAT GAGCTTCCTC
     48f  CATCTACGAC CTTAGCAATG GAGAATTTGT AAGAGGAAAT GAGCTTCCTC

          651                                                  700
Hsu09278  GTCCAATTCA GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT
Hsu76833  GTCCAATTCA GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT
   56f_1  GTCCAATTCA GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT
     49f  GTCCAATTCA GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT
     48f  GTCCAATTCA GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT

          701                                                  750
Hsu09278  GTCTATCAAA ACAATATCTA TTTGAAACAA AGACCAGGAG ATCCACCTTT
Hsu76833  GTCTATCAAA ACAATATCTA TTTGAAACAA AGACCAGGAG ATCCACCTTT
   56f_1  GTCTATCAAA ACAATATCTA TTTGAAACAA AGACCAGGAG ATCCACCTTT
     49f  GTCTATCAAA ACAATATCTA TTTGAAACAA AGACCAGGAG ATCCACCTTT
     48f  GTCTATCAAA ACAATATCTA TTTGAAACAA AGACCAGGAG ATCCACCTTT

          751                                                  800
Hsu09278  TCAAATAACA TTTAATGGAA GAGAAAATAA AATATTTAAT GGAATCCCAG
Hsu76833  TCAAATAACA TTTAATGGAA GAGAAAATAA AATATTTAAT GGAATCCCAG
   56f_1  TCAAATAACA TTTAATGGAA GAGAAAATAA AATATTTAAT GGAATCCCAG
     49f  TCAAATAACA TTTAATGGAA GAGAAAATAA AATATTTAAT GGAATCCCAG
     48f  TCAAATAACA TTTAATGGAA GAGAAAATAA AATATTTAAT GGAATCCCAG

          801                                                  850
Hsu09278  ACTGGGTTTA TGAAGAGGAA ATGCTTCCTA CAAAATATGC TCTCTGGTGG
Hsu76833  ACTGGGTTTA TGAAGAGGAA ATGCTTGCTA CAAAATATGC TCTCTGGTGG
   56f_1  ACTGGGTTTA TGAAGAGGAA ATGCTTGCTA CAAAATATGC TCTCTGGTGG
     49f  ACTGGGTTTA TGAAGAGGAA ATGCTTGCTA CAAAATATGC TCTCTGGTGG
     48f  ACTGGGTTTA TGAAGAGGAA ATGCTTGCTA CAAAATATGC TCTCTGGTGG

          851                                                  900
Hsu09278  TCTCCTAATG GAAAATTTTT GGCATATGCG GAATTTAATG ATAAGGATAT
Hsu76833  TCTCCTAATG GAAAATTTTT GGCATATGCG GAATTTAATG ATACGGATAT
   56f_1  TCTCCTAATG GAAAATTTTT GGCATATGCG GAATTTAATG ATACGGATAT
     49f  TCTCCTAATG GAAAATTTTT GGCATATGCG GAATTTAATG ATACGGATAT
     48f  TCTCCTAATG GAAAATTTTT GGCATATGCG GAATTTAATG ATACGGATAT

          901                                                  950
Hsu09278  ACCAGTTATT GCCTATTCCT ATTATGGCGA TGAACAATAT CCTAGAACAA
Hsu76833  ACCAGTTATT GCCTATTCCT ATTATGGCGA TGAACAATAT CCTAGAACAA
   56f_1  ACCAGTTATT GCCTATTCCT ATTATGGCGA TGAACAATAT CCTAGAACAA
     49f  ACCAGTTATT GCCTATTCCT ATTATGGCGA TGAACAATAT CCTAGAACAA
     48f  ACCAGTTATT GCCTATTCCT ATTATGGCGA TGAACAATAT CCTAGAACAA
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          951                                                  1000
Hsu09278  TAAATATTCC ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTCGGATA
Hsu76833  TAAATATTCC ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTCGGATA
   56f_1  TAAATATTCC ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTCGGATA
     49f  TAAATATTCC ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTCGGATA
     48f  TAAATATTCC ATACCCAAAG GCTGGAGCTA AGAATCCCGT TGTTCGGATA

          1001                                                 1050
Hsu09278  TTTATTATCG ATACCACTTA CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC
Hsu76833  TTTATTATCG ATACCACTTA CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC
   56f_1  TTTATTATCG ATACCACTTA CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC
     49f  TTTATTATCG ATACCACTTA CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC
     48f  TTTATTATCG ATACCACTTA CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC

          1051                                                 1100
Hsu09278  TGTTCCAGCA ATGATAGCCT CAAGTGATTA TTATTTCAGT TGGCTCACGT
Hsu76833  TGTTCCAGCA ATGATAGCCT CAAGTGATTA TTATTTCAGT TGGCTCACGT
   56f_1  TGTTCCAGCA ATGATAGCCT CAAGTGATTA TTATTTCAGT TGGCTCACGT
     49f  TGTTCCAGCA ATGATAGCCT CAAGTGATTA TTATTTCAGT TGGCTCACGT
     48f  TGTTCCAGCA ATGATAGCCT CAAGTGATTA TTATTTCAGT TGGCTCACGT

          1101                                                 1150
Hsu09278  GGGTTACTGA TGAACGAGTA TGTTTGCAGT GGCTAAAAAG AGTCCAGAAT
Hsu76833  GGGTTACTGA TGAACGAGTA TGTTTGCAGT GGCTAAAAAG AGTCCAGAAT
   56f_1  GGGTTACTGA TGAACGAGTA TGTTTGCAGT GGCTAAAAAG AGTCCAGAAT
     49f  GGGTTACTGA TGAACGAGTA TGTTTGCAGT GGCTAAAAAG AGTCCAGAAT
     48f  GGGTTACTGA TGAACGAGTA TGTTTGCAGT GGCTAAAAAG AGTCCAGAAT

          1151                                                 1200
Hsu09278  GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGGA
Hsu76833  GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGGA
   56f_1  GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC GGACATGGGA
     49f  GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGGA
     48f  GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGGA

          1201                                                 1250
Hsu09278  TTGTCCAAAG ACCCAGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG
Hsu76833  TTGTCCAAAG ACCCAGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG
   56f_1  TTGTCCAAAG ACCCAGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG
     49f  TTGTCCAAAG ACCCAGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG
     48f  TTGTCCAAAG .......... .......... .......... ..........

          1251                                                 1300
Hsu09278  GTGGATTCTT TGTTTCAAGA CCAGTTTTCA GCTATGATGC CATTTCGTAC
Hsu76833  GTGGATTCTT TGTTTCAACA CCAGTTTTCA GCTATGATGC CATTTCGTAC
   56f_1  GTGGATTCTT TGTTTCAACA CCAGTTTTCA GCTATGATGC CATTTCGTAC
     49f  GTGGATTCTT TGTTTCAACA CCAGTTTTCA GCTATGATGC CATTTCGTAC
     48f  .....TTCTT TGTTTCAACA CCAGTTTTCA GCTATGATGC CATTTCGTAC
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          1301                                                 1350
Hsu09278  TACAAAATAT TTAGTGACAA GGATGGCTAC AAACATATTC ACTATATCAA
Hsu76833  TACAAAATAT TTAGTGACAA GGATGGCTAC AAACATATTC ACTATATCAA
   56f_1  TACAAAATAT TTAGTGACAA GGATGGCTAC AAACATATTC ACTATATCAA
     49f  TACAAAATAT TTAGTGACAA GGATGGCTAC AAACATATTC ACTATATCAA
     48f  TACAAAATAT TTAGTGACAA GGATGGCTAC AAACATATTC ACTATATCAA

          1351                                                 1400
Hsu09278  AGACACTGTG GAAAATGCTA TTCAAATTAC AAGTGGCAAG TGGGAGGCCA
Hsu76833  AGACACTGTG GAAAATGCTA TTCAAATTAC AAGTGGCAAG TGGGAGGCCA
   56f_1  AGACACTGTG GAAAATGCTA TTCAAATTAC AAGTGGCAAG TGGGAGGCCA
     49f  AGACACTGTG GAAAATGCTA TTCAAATTAC AAGTGGCAAG TGGGAGGCCA
     48f  AGACACTGTG GAAAATGCTA TTCAAATTAC AAGTGGCAAG TGGGAGGCCA

          1401                                                 1450
Hsu09278  TAAATATATT CAGAGTAACA CAGGATTCAC TGTTTTATTC TAGCAATGAA
Hsu76833  TAAATATATT CAGAGTAACA CAGGATTCAC TGTTTTATTC TAGCAATGAA
   56f_1  TAAATATATT CAGAGTAACA CAGGATTCAC TGTTTTATTC TAGCAATGAA
     49f  TAAATATATT CAGAGTAGCA CAGGATTCAC TGTTTTATTC TAGCAATGAA
     48f  TAAATATATT CAGAGTAACA CAGGATTCAC TGTTTTATTC TAGCAATGAA

          1451                                                 1500
Hsu09278  TTTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAG
Hsu76833  TTTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAG
   56f_1  TTTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAG
     49f  TTTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAG
     48f  TTTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAG

          1501                                                 1550
Hsu09278  CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAGGT
Hsu76833  CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAGGT
   56f_1  CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCGTCTAAGG AAAGAAAGGT
     49f  CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAGGT
     48f  CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAGGT

          1551                                                 1600
Hsu09278  GCCAATATTA CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT
Hsu76833  GCCAATATTA CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT
   56f_1  GCCAATATTA CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT
     49f  GCCAATATTA CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT
     48f  GCCAATATTA CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT

          1601                                                 1650
Hsu09278  GTCTGCTACG GCCCAGGCAT CCCCATTTCC ACCCTTCATG ATGGACGCAC
Hsu76833  GTCTGCTACG GCCCAGGCAT CCCCATTTCC ACCCTTCATG ATGGACGCAC
   56f_1  GTCTGCTACG GCCCAGGCAT CCCCATTTCC ACCCTTCATG ATGGACGCAC
     49f  GTCTGCTACG GCCCAGGCAT CCCCATTTCC ACCCTTCATG ATGGACGCAC
     48f  GTCTGCTACG GCCCAGGCAT CCCCATTTCC ACCCTTCATG ATGGACGCAC

          1651                                                 1700
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Hsu09278  TGATCAAGAA ATTAAAATCC TGGAAGAAAA CAAGGAATTG GAAAATGCTT
Hsu76833  TGATCAAGAA ATTAAAATCC TGGAAGAAAA CAAGGAATTG GAAAATGCTT
   56f_1  TGATCAAGAA ATTAAAATCC TGGAAGAAAA CAAGGAATTG GAAAATGCTT
     49f  TGATCAAGAA ATTAAAATCC TGGAAGAAAA CAAGGAATTG GAAAATGCTT
     48f  TGATCAAGAA ATTAAAATCC TGGAAGGAAA CAAGGAATTG GAAAATGCTT

          1701                                                1750
Hsu09278  TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT TGAAGTAGAT
Hsu76833  TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT TGAAGTAGAT
   56f_1  TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT TGAAGTAGAT
     49f  TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT TGAAGTAGAT
     48f  TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT TGAAGTAGAT

          1751                                                1800
Hsu09278  GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC
Hsu76833  GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC
   56f_1  GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC
     49f  GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC
     48f  GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC

          1801                                                1850
Hsu09278  AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGCAGTCAGA
Hsu76833  AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGCAGTCAGA
   56f_1  AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGCAGTCAGA
     49f  AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGCAGTCAGA
     48f  AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGCAGTCAGA

          1851                                                1900
Hsu09278  GTGTAAGGTC TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG
Hsu76833  GTGTAAGGTC TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG
   56f_1  GTGTAAGGTC TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG
     49f  GTGTAAGGTC TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG
     48f  GTGTAAGGTC TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG

          1901                                                1950
Hsu09278  GAAGGGATGG TCATTGCCTT GGTGGATGGT CGAGGAACAG CTTTCCAAGG
Hsu76833  GAAGGGATGG TCATTGCCTT GGTGGATGGT CGAGGAACAG CTTTCCAAGG
   56f_1  GAAGGGATGG TCATTGCCTT GGTGGATGGT CGAGGAACAG CTTTCCAAGG
     49f  GAAGGGATGG TCATTGCCTT GGTGGATGGT CGAGGAACAG CTTTCCAAGG
     48f  GAAGGGATGG TCATTGCCTT GGTGGATGGT CGAGGAACAG CTTTCCAAGG

          1951                                                2000
Hsu09278  TGACAAACTC CTCTATGCAG TGTATCGAAA GCTGGGTGTT TATGAAGTTG
Hsu76833  TGACAAACTC CTCTATGCAG TGTATCGAAA GCTGGGTGTT TATGAAGTTG
   56f_1  TGACAAACTC CTCTATGCAG TGTATCGAAA GCTGGGTGTT TATGAAGTTG
     49f  TGACAAACTC CTCTATGCAG TGTATCGAAA GCTGGGTGTT TATGAAGTTG
     48f  TGACAAACTC CTCTATGCAG TGTATCGAAA GCTGGGTGTT TATGAAGTTG

          2001                                                2050
Hsu09278  AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG TTTCATTGAT
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Hsu76833  AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG TTTCATTGAT
   56f_1  AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG TTTCATTGAT
     49f  AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG TTTCATTGAT
     48f  AAGACCAGAT TACAGCTGTC AGAAAATTCA TAGAAATGGG TTTCATTGAT

          2051                                              2100
Hsu09278  GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTTTCATC
Hsu76833  GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTTTCATC
   56f_1  GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTTTCATC
     49f  GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTCTCATC
     48f  GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTTTCATC

          2101                                              2150
Hsu09278  ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG
Hsu76833  ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG
   56f_1  ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG
     49f  ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG
     48f  ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG

          2151                                              2200
Hsu09278  CTCCAGTCTC CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTC
Hsu76833  CTCCAGTCTC CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTC
   56f_1  CTCCAGTCTC CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTC
     49f  CTCCAGTCTC CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTC
     48f  CTCCAGTCTC CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTC

          2201                                              2250
Hsu09278  ATGGGTCTCC CAACAAAGGA TGATAATCTT GAGCACTATA AGAATTCAAC
Hsu76833  ATGGGTCTCC CAACAAAGGA TGATAATCTT GAGCACTATA AGAATTCAAC
   56f_1  ATGGGTCTCC CAACAAAGGA TGATAATCTT GAGCACTATA AGAATTCAAC
     49f  ATGGGTCTCC CAACAAAGGA TGATAATCTT GAGCACTATA AGAATTCAAC
     48f  ATGGGTCTCC CAACAAAGGA TGATAATCTT GAGCACTATA AGAATTCAAC

          2251                                              2300
Hsu09278  TGTGATGGCA AGAGCAGAAT ATTTCAGAAA TGTAGACTAT CTTCTCATCC
Hsu76833  TGTGATGGCA AGAGCAGAAT ATTTCAGAAA TGTAGACTAT CTTCTCATCC
   56f_1  TGTGATGGCA AGAGCAGAAT ATTTCAGAAA TGTAGACTAT CTTCTCATCC
     49f  TGTGATGGCA AGAGCAGAAT ATTTCAGAAA TGTAGACTAT CTTCTCATCC
     48f  TGTGATGGCA AGAGCAGAAT ATTTCAGAAA TGTAGACTAT CTTCTCATCC

          2301                                              2350
Hsu09278  ACGGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC ACAGATTGCT
Hsu76833  ACGGAACAGC AGATGATAAT GTGCACTTTC AGAACTCAGC ACAGATTGCT
   56f_1  ACGGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC ACAGATTGCT
     49f  ACGGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC ACAGATTGCT
     48f  ACGGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC ACAGATTGCT

          2351                                              2400
Hsu09278  AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACTCTGA
Hsu76833  AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACTCTGA
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   56f_1  AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACCCTGA
     49f  AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACTCTGA
     48f  AAGGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACTCTGA

          2401                                                2450
Hsu09278  CCAGAACCAC GGCTTATCCG GCCTGTCCAC GAACCACTTA TACACCCACA
Hsu76833  CCAGAACCAC GGCTTATCCG GCCTGTCCAC GAACCACTTA TACACCCACA
   56f_1  CCAGAACCAC GGCTTATCCG GCCTGTCCAC GAACCACTTA TACACCCACA
     49f  CCAGAACCAC GGCTTGTCCG GCCTGTCCAC GAACCACTTA TACACCCACA
     48f  CCAGAACCAC GGCTTATCCG GCCTGTCCAC GAACCACTTA TACACCCACA

          2451                                                2500
Hsu09278  TGACCCACTT CCTAAAGCAG TGTTTCTCTT TGTCAGACTA AAAACGATGC
Hsu76833  TGACCCACTT CCTAAAGCAG TGTTTCTCTT TGTCAGACTA AAAACGATGC
   56f_1  TGACCCACTT CCTAAAGCAG TGTTTCTCTT TGTCAGACTA AAAACGATGC
     49f  TGACCCACTT CCTAAAGCAG TGTTTCTCTT TGTCAGACTA AAAACGATGC
     48f  TGACCCACTT CCTAAAGCAG TGTTTCTCTT TGTCAGACTA AAAACGATGC

          2501                                                2550
Hsu09278  AGATGCAAGC CTGTATCAGA ATCTGAAAAC CTTATATAAA CCCCTCAGAC
Hsu76833  AGATGCAAGC CTGTATCAGA ATCTGA~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   56f_1  AGATGCAAGC CTGTATCAGA ATCTGAAAGG GCGAATTCCA GCACACTGGC
     49f  AGATGCAAGC CTGTATCAGA AT...AAGGG CGAATTCCAA GCACACTGGC
     48f  AGATGCAAGC CTGTATCAGA ATCTGAAAGG GCGAATTCCA GCACACTGGC

          2551                                                2600
Hsu09278  AGTTTGCTTA TTTTATTTTT TATGTTGTAA AATGCTAGTA TAAACAAACA
Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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     49f  GGCCGTTACT AAGTGGATCC GAGCTCGGTA CCAAGCTTGG CGTAATCATG
     48f  GGCCGTTACT AGTGGAT~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2601                                                2650
Hsu09278  AATTAATGTT GTTCTAAAGG CTGTTAAAAA AAAGATGAGG ACTCAGAAGT
Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   56f_1  GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA
     49f  GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA
     48f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2651                                                2700
Hsu09278  TCAAGCTAAA TATTGTTTAC ATTTTCTGGT ACTCTGTGAA AGAAGAGAAA
Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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     49f  ACATACGAGC CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG
     48f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2701                                                2750
Hsu09278  AGGGAGTCAT GCATTTTGCT TTGGACACAG TGTTTTATCA CCTGTTCATT
Hsu76833  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   56f_1  AGCCAACTCA CATTAATTGC GTTGCGCTCA ACTGGCCCGC TTTTCAATCC
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    49f  AGCTAACTCA CATTAA~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
    48f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

         2751                                                2800
Hsu09278 TGAAGAAAAA TAATAAAGTC AGAAGTTCAA AAAAAAAAAA AAAAAAAAAA
Hsu76833 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  56f_1  GGGAAAACTG TCCTTGCCAA CTGCATTAAT GAATCGGCCA ACGCCCCGGG
    49f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
    48f  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

         2801       2814
Hsu09278 AAAGCGGCCG CTCG
Hsu76833 ~~~~~~~~~~ ~~~~
  56f_1  GAAAAGC~~~ ~~~~
    49f  ~~~~~~~~~~ ~~~~
    48f  ~~~~~~~~~~ ~~~~
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Accession/ID:

====================General comments====================

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to:
 2366
Accession/ID:

====================General comments====================

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151 QYLCWSPVGSKLAYVYQNNIYLKQRPGDPPFQITFNGRENKIFNGIPDWV 200
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Clin. Exp. Metastasis, 1998, **16**, 501–512

Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases

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Breast cancer cell lines vary in invasive behavior and one highly invasive cell line (MDA-MB-231) proteolytically degrades extracellular matrix with invadopodia (Thompson *et al.* 1992, *J Cell Physiol*, 150, 534–44; Chen *et al.* 1994, *Breast Cancer Res Treat*, 31, 217–26). Invadopodial proteolysis of extracellular matrix is thought to be necessary for invasion; however, this has not been demonstrated directly. To obtain such evidence, normal (HBL-100) and malignant (MCF-7, MDA-MB-231) breast cells were evaluated for invadopodial proteolysis of extracellular matrix and invasive behavior. We report that invadopodial proteolysis of immobilized fibronectin is positively correlated with invasion of cells into type I collagen gels. Moreover, reducing the proteolytic activity of invadopodia with the metalloproteinase inhibitor, batimastat (BB-94), also decreases invasion indicating that breast cancer cell invasion is dependent upon proteolytically active invadopodia.

Keywords: (BB-94) batimastat, fibronectin, invadopodia, invasion, metastasis, MMP

Introduction

Invasive tumor cells have increased levels of a variety of extracellular matrix-degrading proteases that allow them to traverse complex basement membrane and stromal matrices (reviewed in [1–3]). Generally, the matrix-degrading proteases are secreted by tumor and stromal cells as inactive zymogens that require association with the tumor cell surface to become activated and capable of degrading extracellular matrix [1,2]. The cellular sites of abnormally high extracellular matrix-degrading activity can be investigated by growing invasive cells on monolayers of fluorescently labeled extracellular matrix proteins. Fluorescence-negative regions underneath the invasive cells are observed that indicate the positions where matrix proteolysis has occurred [4,5]. The proteolytic activity is discretely focused and corresponds to areas where plasma membrane protrusions termed 'invadopodia' extend from cell surfaces and contact the matrix [4, 6]. Invadopodia can degrade multiple extracellular matrix proteins including intact fibronectin-rich matrices produced by fibroblasts, fibronectin, laminin, type IV collagen, and type I collagen [5]. A variety of invasive cells exhibit invadopodia including human melanoma cells [7], transformed chicken fibroblasts [4–6], and human breast cancer cells [8,9]. Invadopodial proteolysis of extracellular matrix is thought to facilitate tumor cell invasion into extracellular matrix [3,6,10].

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Matrix metalloproteinases (MMPs) are important mediators of invadopodial degradation of extracellular matrix because an inhibitor of MMPs (NP-20) decreases invadopodial degradation of type I collagen by Rous sarcoma virus-transformed chicken embryo fibroblasts [11]. MMPs are a family of structurally related enzymes that together can degrade all components of the extracellular matrix and are known to be important in tumor cell invasion [1,2]. MMPs are synthesized as inactive proenzymes that require cleavage of the pro-peptide for activation of their proteolytic activities. Proteolytically active MMP-2 (gelatinase A) and MT1-MMP (membrane type 1-matrix metalloproteinase or MMP-14) are concentrated on invadopodial membranes suggesting that these enzymes are important mediators of invadopodial extracellular matrix degradation [11,12]. MT1-MMP is activated by furin-like enzymes prior to its expression on the cell surface [13,14] where it serves as a potent activator of latent MMP-2 [15,16]. Given the wide range of extracellular matrix substrates degraded by invadopodia, other proteases including other MMPs are likely to have a role in proteolysis of extracellular matrix by invadopodia.

This study was performed to determine if invadopodial proteolysis of extracellular matrix plays an important role in facilitating human breast cell invasion. Previously, it was shown that the invasive MDA-MB-231 human breast cancer cells use invadopodia to degrade films of fluorescent extracellular matrix molecules covalently attached to glutaraldehyde crosslinked gelatin [8,9]. Independently, others have shown that human breast cancer cell lines vary in their ability to invade into Matrigel and that human breast cancer cell lines vary in local invasion through the peritoneum when injected into the mammary fat pads of nude mice [17]. MDA-MB-231 cells were identified as invasive in both assays [8,17]. In contrast, MCF-7 human breast cancer cells were moderately invasive through Matrigel in Boyden chamber assays and, although these cells formed tumors when injected into the mammary fat pads of nude mice, the tumor cells did not invade through the peritoneum [17]. Although invadopodial proteolysis of extracellular matrix and invasiveness have been assessed separately, there has not been a direct evaluation of the role of invadopodial proteolysis of extracellular matrix in invasion. Here we correlate the HBL-100, MCF-7, and MDA-MB-231 human breast cell lines for invadopodial function as determined by degradation of fluorescently labeled or radiolabeled fibronectin and for invasiveness using type I collagen gels. The MMP inhibitor batimastat was used to inhibit the function of invadopodia to thereby investigate the role of invadopodia in breast cancer cell invasion. We show that invadopodial proteolysis of extracellular matrix facilitates human breast cancer cell invasion and is mediated by MMPs.

Materials and methods

Cell culture

Human breast adenocarcinoma cell lines MDA-MB-231, MCF-7, and the normal breast cell line HBL-100 were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in Eagle's minimal essential medium (Gibco-BRL, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 10 μg/ml bovine insulin (Sigma, St. Louis, MO), 10 μg/ml glutamine (Gibco-BRL, Gaithersburg, MD), 1% penicillin-streptomycin (Gibco-BRL) 2×10^{-8} mM β-estradiol (Sigma) and maintained at 37°C in 95% air and 5% CO_2. The FBS used for growing cells was either complete (Figure 4), depleted of MMPs and tissue inhibitors of metalloproteinases (TIMPs; Figures 1 and 3A) or depleted of MMPs, TIMPs and plasmin/plasminogen (Table 1, Figures 2, 3B and 5) as described below.

Fluorescent fibronectin substrates for determining invadopodial proteolysis of extracellular matrix

This assay relies on growing cells on fluorescein isothiocyanate-labeled fibronectin that is covalently bound to a glutaraldehyde-crosslinked gelatin film attached to a glass coverslip. Fluorescence-negative regions underneath cells represent areas where the extracellular matrix has been degraded [4,5]. Human plasma fibronectin (Becton Dickinson Labware, Medford, MA) was coupled to fluorescein isothiocyanate (Research Organics, Cleveland, OH) according to the manufacturer's instructions. The assays reported here were allowed to proceed for 24 or 72 h at 37°C in 95% air and 5% CO_2 prior to fixation and preparation for fluorescence microscopy.

Fluorescence microscopy

The cells were fixed, stained with rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) as described earlier [5]. The cells were observed using ×40, ×60 and ×100 objectives of a Nikon LABOPHOT microscope equipped for epifluorescence photomicroscopy and images were recorded using Ilford HP5 400 ASA black and white film. To quantify invadopodial matrix proteolysis, 10 micro-

scopic fields on each coverslip were randomly selected and the fluorescence of the fibronectin film visualized with the × 40 objective and scored for the presence or absence of matrix proteolysis as judged by the presence of focal fluorescence negative spots in areas where the matrix was degraded [4,5,8]. The results are presented as the average number of fields positive for matrix degradation per 10 fields examined.

[^{125}I]fibronectin substrates for determining invadopodial proteolysis of extracellular matrix

This second invadopodial proteolysis assay utilized [^{125}I]fibronectin as the substrate and protease-depleted FBS in the growth medium. The assay involves: iodination of the fibronectin, coupling the [^{125}I]fibronectin to crosslinked gelatin films, determination of [^{125}I]fibronectin bound to the substrate, extensive washing to remove free [^{125}I]fibronectin, and determination of invadopodial proteolysis.

Removal of interfering extracellular matrix-degrading proteases from FBS

Gelatin Sepharose chromatography was used to remove MMPs and TIMPs from FBS [18,19]. A 10 ml gelatin-Sepharose (Pharmacia Biotech, Uppsala, Sweden) was equilibrated with the binding buffer 20 mM Tris, 0.5 M NaCl, 1.0 mM $CaCl_2$, 10% glycerol (v/v), 0.05% BRIJ-35 (v/v), 0.02% NaN_3 (v/v), pH 7.6. MMPs and TIMPs in FBS (heat inactivated) were bound to the column by loading the serum (50 ml) onto the column at 35 ml/h. The void volume was discarded, and the flow-through, depleted of MMPs and TIMPs but containing the other serum components, was collected and sterile filtered. Gelatin zymography of breast cancer cell growth medium made 10% (v/v) with respect to FBS revealed no MMP activity (not shown).

For the batimastat inhibitor studies (batimastat kindly provided by British Biotech Pharmaceuticals, Ltd, Oxford, UK), the FBS was depleted of MMPs, TIMPs, and the broad spectrum protease, plasmin/plasminogen, that is abundant in serum. MMPs and TIMPs were removed as described and then lysine Sepharose was used to remove the plasmin/plasminogen [20]. A 10 ml lysine Sepharose column (Pharmacia Biotech) was packed and equilibrated with the same binding buffer as described above. The MMP/TIMP-depleted FBS (50 ml) was loaded onto the column at 35 ml/h. The MMP/TIMP and plasminogen/plasmin-depleted serum in the flow-through was collected and sterile filtered. Immunoblot analysis of breast cancer cell growth medium made 10% (v/v) with respect to MMP/TIMP and plasminogen/plasmin-depleted FBS revealed no plasminogen or plasmin immunoreactivity with a goat IgG directed against bovine plasminogen (American Diagnostics, Greenwich, CT) (not shown).

Iodination of fibronectin, coupling to crosslinked gelatin and determination of cpm [^{125}I]fibronectin bound to the substrate

Fibronectin (50 μg) was iodinated with 0.5 mCi ^{125}I using chloramine T (2 mg/ml) as described [21]. [^{125}I]-fibronectin was separated from free ^{125}I by gel filtration using an extracellulose G5 column (Pierce, Rockford, IL) that had been equilibrated in 1 mg/ml bovine serum albumin (BSA) and phosphate buffered serum (PBS). An equivalent amount of [^{125}I]-fibronectin in 200 μl was coupled to the glutaraldehyde crosslinked gelatin film coating 15 mm glass round coverslips as previously described for fluorescent fibronectin [4,5,22]. The level of [^{125}I]fibronectin used in independent experiments varied from 2×10^6 cpm to 10^{10} cpm. The ^{125}I-labeled fibronectin was allowed to bind to the coverslips for at least 12 h. At the end of coupling, 1 μl of the coupling fluid was counted and the volume of coupling fluid was measured to determine the amount of [^{125}I]fibronectin that did and did not bind to each coverslip.

The [^{125}I]fibronectin-coupled crosslinked gelatin films were subjected to a series of washes designed to remove any free or weakly bound [^{125}I]fibronectin and to block exposed aldehyde groups. The washes included 3 ml of the following solutions: 70% ethanol (1×5 min, 22°C), PBS (3×5 min, 22°C), growth medium containing 10% MMP/TIMP and plasminogen/plasmin-depleted FBS (1×3 h, 37°C), PBS (2×5 min, 22°C), and serum-free growth medium (1×24 h, 37°C). A sample (10 μl) was taken to determine the level of [^{125}I]fibronectin in each wash. The total [^{125}I]fibronectin that did not bind each coverslip was determined by adding the radioactivity (cpm) in the unbound fraction to the sum of the radioactivity (cpm) released by all of the washes. Then the amount of [^{125}I]fibronectin bound to each coverslip was determined by subtracting the total unbound radioactivity from the radioactivity originally added to the coverslip.

Determination of invadopodial matrix proteolysis using immobilized [^{125}I]fibronectin

Human breast cells were harvested using trypsin–EDTA, diluted with growth medium containing 10% protease-depleted FBS and washed three times with sterile PBS. Coverslips were placed in the wells of a 6-well culture plate. Cells (1×10^5) were placed onto each coverslip in 200 μl of medium and allowed to attach to the [^{125}I]fibronectin for 1 h at 37°C. Then

2.8 ml of growth medium containing 10% MMP/TIMP and plasminogen/plasmin-depleted FBS containing no additives, DMSO, or 10 μM batimastat in DMSO was added to achieve a final volume of 3 ml. The plates were placed back into the incubator and 50 μl aliquots were taken from each well under sterile conditions and counted at 3, 24, 48, 72 and 96 h. At each time point, 50 μl of growth media containing 10 % protease-depleted FBS were put back into each well to maintain the 3 ml volume of the assay. The radioactivity of the aliquots was determined with a Packard gamma counter.

The total amount of ^{125}I released into the media for each time point was calculated and added to the sum of the radioactivity in the 50 μl aliquots from prior time points. This value was divided by the total cpm [^{125}I]fibronectin bound to that coverslip and multiplied by 100 to give the percentage of total bound ^{125}I released into the media.

Cell viabilities

The cytotoxicity of 72 h exposure to batimastat and DMSO was investigated by performing trypan blue exclusion assays as described by others [23].

Gelatin zymography

Gelatin zymography was performed essentially as described by Heussen and Dowdle [24] with 1 mg/ml gelatin co-polymerized into SDS–PAGE that was 10% (w/v) with respect to acrylamide.

Conditioned growth medium

To determine the protease activities released into the medium by human breast cancer cells and to investigate the inhibitory effects of batimastat on those proteases, MDA-MB-231 cells were grown to high density (7×10^5 cells/ml) in growth medium containing 10% (v/v) FBS in 75 cm^2 flasks. Complete growth medium was removed and the cells washed three times with 10 ml sterile PBS. After removing the final wash, the cells were incubated in serum-free minimal essential media for 48 h at 37°C in 5% CO_2. The conditioned medium was collected, cells and debris removed by centrifugation (5000 ***g***, 5 min), and concentrated 10 times using centricon 30 filter concentrators (Amicon, Inc., Beverly, MA). The concentrated conditioned medium was tested for proteases in the presence or absence of 10 μM batimastat by zymography.

Invasion assay

Type I collagen gel invasion assays were performed in 24-well plates as described by Liebersbach and Sanderson [25] except that the assays were allowed to proceed for 72 h. Quantification of the percentage of invading cells was achieved by first determining the number of non-invading cells that could be removed from the top of the gel with trypsin-EDTA and then counting the invading cells that were released from within the type I collagen gel by extensive collagenase digestion as described by Liebersbach and Sanderson [25]. The leading front of invasion was defined as the point where the two most distantly migrating breast cancer cells were simultaneously in focus in one field under ×200 magnification. After 72 h, each gel was searched in a Z pattern and the mean distance ± standard error of the mean was determined using the calibrated micrometer of a Nikon inverted phase contrast microscope. Within experiments, duplicate assays were performed for each cell line and each experiment was repeated at least two times.

Results

Invadopodial extracellular matrix degrading activities of human breast cell lines

The MDA-MB-231 human breast cancer cells are proteolytically active and degrade extracellular matrix substrates by an invadopodial-dependent mechanism [8,9]. Other work has shown that human breast cancer cell lines differ in invasive behavior as determined *in vitro* using a Boyden chamber invasion assay with Matrigel as the barrier to invasion and the invasiveness observed *in vitro* correlates closely with *in vivo* invasive behavior observed using the nude mouse model [17]. These workers identified the MDA-MB-231 cell line as one of the most invasive. The purpose of this study was to determine the role of invadopodial proteolysis of extracellular matrix in the invasion process.

The extracellular matrix-degrading activity of the human breast cell lines was investigated by growing the cells on fluorescently labeled fibronectin that was covalently coupled to a glutaraldehyde-crosslinked gelatin film. HBL-100 normal human breast cells do not degrade the matrix and the fibronectin substrate remains intact and uniformly fluorescent underneath the cells (Figure 1A, B). MCF-7 human breast cancer cells reveal limited degradation of the fluorescent-fibronectin film, with one microscopic field in ten having the spots of decreased fluorescence that are indicative of matrix proteolysis (not shown). The MDA-MB-231 breast cancer cell line reveals extensive degradation of the fluorescent fibronectin films, with five microscopic fields in ten having evidence of matrix degradation (Figure 1C, D).

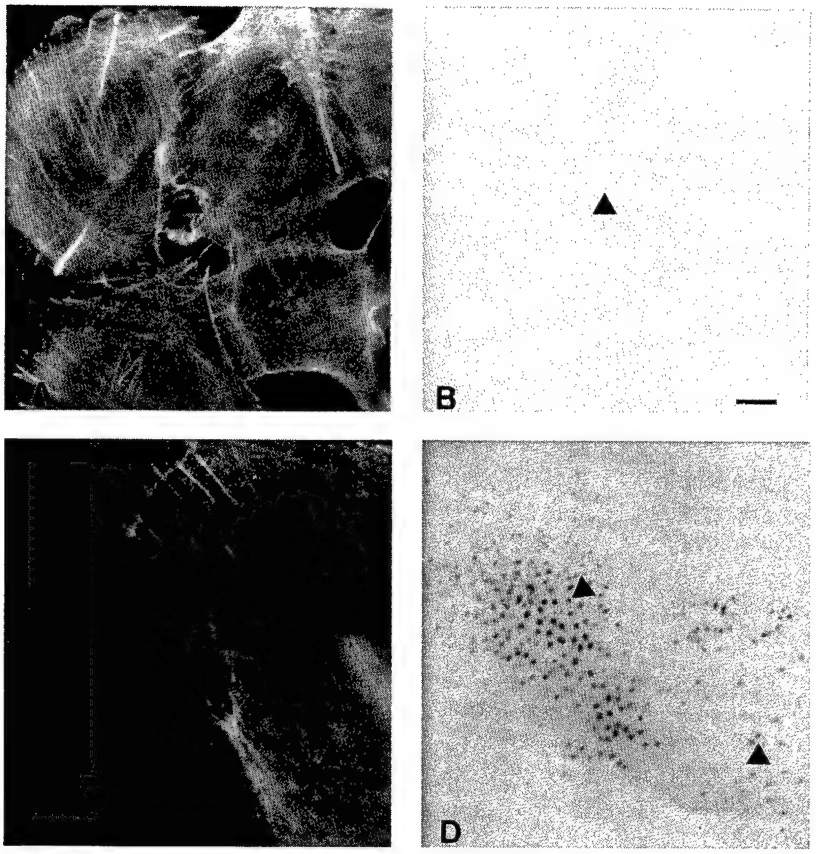

Figure 1. Normal HBL-100 human breast cells do not degrade fibronectin but malignant MDA-MB-231 human breast cancer cells degrade fibronectin. Normal HBL-100 cells are visualized by rhodamine phalloidin fluorescence (A). The fibronectin substrate in the same microscopic field is visualized by fluorescein–fibronectin fluorescence respectively (B). Arrowheads point to identical locations in the microscopic fields. Malignant MDA-MB-231 human breast cells (C) degrade fibronectin that is covalently linked to the substrate (D). Arrowheads point to identical locations in the same microscopic field. The observed fluorescence-negative spots where the fibronectin has been removed are characteristic of invadopodia-mediated proteolysis (arrowheads: D). Cells were grown on the fibronectin films for 72 h. Bar = 10 μm.

The fact that MMP-2 and MT1-MMP are concentrated on invadopodia [11,12] led us to investigate the effect of MMP inhibition on proteolysis of extracellular matrix by MDA-MB-231 cells. Batimastat (Mr 477) is a broad spectrum MMP inhibitor that mimics one part of the principle MMP-cleavage site in collagen. Batimastat was used because it has nM IC_{50} values for inhibition of several MMPs including: MMP-1 (interstitial collagenase), MMP-3 (stromelysin), MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-7 (matrilysin) [26]. Batimastat also blocks the activation of MMP-2, presumably by inhibition of MMP-14 (MT1-MMP) [26]. Batimastat does not inhibit metalloproteinases such as angiotensin-converting enzyme and enkephalinase nor does it inhibit other proteases implicated in tumor cell invasion such as plasmin, urokinase-type plasminogen activator, and cathepsins (personal communication, Peter D. Brown, British Biotech Pharmaceuticals, Ltd, Oxford, UK). Other desirable properties of batimastat include low cytotoxicity and a minimal cytostatic effect [26,27]. Moreover, cells treated with batimastat exhibit normal migration in chemotactic assays, indicating that the adhesive and motile machinery of cells is not affected by batimastat [26].

Batimastat was used to investigate the effect of MMP inhibition on extracellular matrix proteolysis by invadopodia. Batimastat completely inhibited invadopodial degradation of fluorescent fibronectin films by MDA-MB-231 breast cancer cells (Figure 2C–F). Invadopodial degradation of extracellular matrix was apparent underneath the untreated MDA-MB-231 cells (Figure 2A, B). Neither the DMSO vehicle nor batimastat were toxic to the cells during incubation periods up to 72 h or at any of the batimastat concentrations used in this study as judged by trypan blue exclusion assays.

To quantify the extracellular matrix proteolysis, normal HBL-100 and malignant MDA-MB-231 breast cells were grown on immobilized [^{125}I]-fibronectin and the radioactivity released from the substrate by the proteolytic action of the cells was determined. MDA-MB-231 cells release 11-fold more [^{125}I]fibronectin degradation products from the substrate than HBL-100 cells after 72 h (Figure 3A). The effect of the batimastat on the proteolysis of extracellular matrix by the MDA-MB-231 breast cancer cell line was investigated. Batimastat reduced the release of [^{125}I]fibronectin by MDA-MB-231 human breast cancer cells to below that released by the

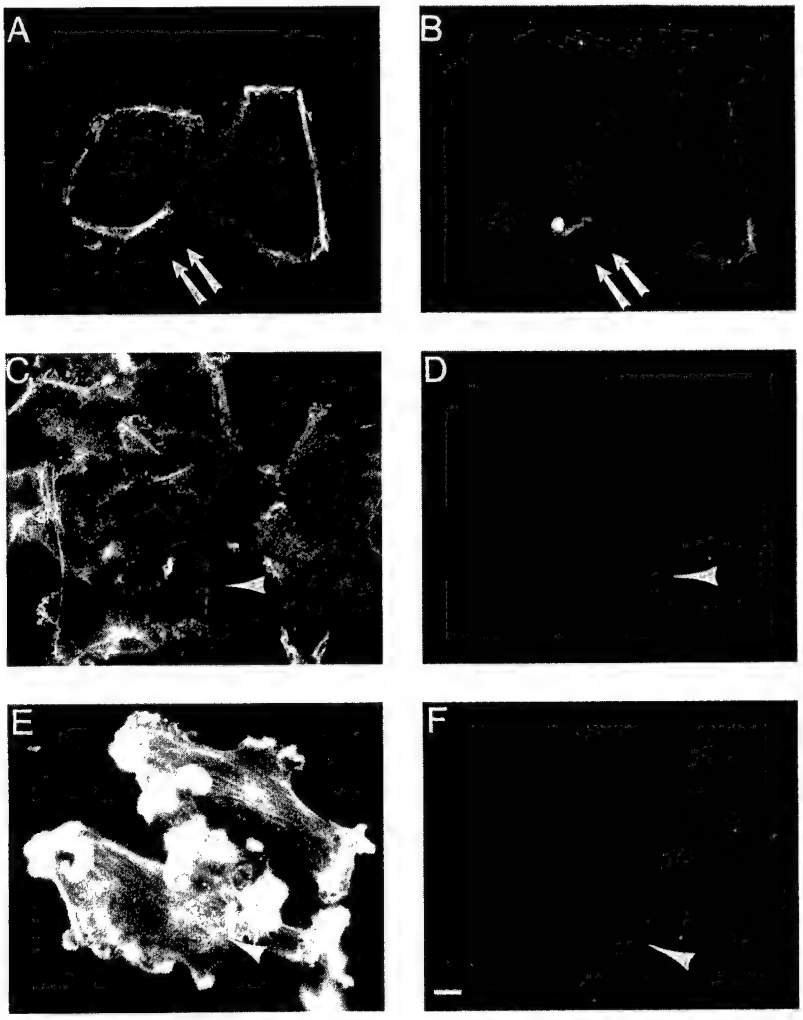

Figure 2. Inhibition of MDA-MB-231 invadopodial matrix degradation by batimastat. Cells (A, C, E) and underlying substrates (B, D, F) in the same microscopic fields (A:B, C:D, and E:F) are visualized for rhodamine phalloidin (cells) and fluorescein–fibronectin (substrates) after growing for 24 h. Proteolytic degradation of fluorescent fibronectin substrates associated with invadopodia is detected in control cells (arrows: A and B) but not in cells treated with 0.1 μM (C and D) or 1.0 μM batimastat (E and F). These substrates had less fluorescent intensity than those used for Figure 1. Bar = 10 μm.

no-cell control (Figure 3B). The release of [^{125}I]fibronectin by MDA-MB-231 cells was only slightly reduced by the DMSO vehicle as compared to untreated cells (Figure 3B). Although it was not cytotoxic, batimastat was active as an inhibitor of MMPs. Batimastat inhibited the 92 and 72 kDa gelatinase activities released into serum-free medium by MDA-MB-231 cells (Figure 3C). The complete inhibition of MDA-MB-231 proteolysis of [^{125}I]fibronectin by batimastat suggests that virtually all of the observed invadopodial proteolysis of extracellular matrix by MDA-MB-231 cells is mediated by MMPs.

Invadopodial extracellular matrix degrading activity correlates with invasion potential of human breast cell lines

Invasion was evaluated by seeding the cells on top of a native type I collagen gel and allowing them to interact with the gel for 72 h (Figure 4). The percentage of cells that invaded into the type I collagen gels after 72 h was determined (Figure 4A). The HBL-100 normal human breast cell line is not invasive in this assay (Figure 4A, H100). The MCF-7 human breast cancer cell line is somewhat invasive (Figure 4A, MCF7) and the MDA-MB-231 human breast cancer cell line is the most invasive in this assay (Figure 4A, M231).

The degree of invasiveness was also reflected in measurements of the leading front of invasion. The leading front of invasion is defined as the deepest level in the gel where at least two cells are simultaneously in focus and is determined at the end of the experiment using the calibrated fine focus of an inverted microscope (Figure 4B). The HBL-100 normal human breast cell line did not invade and the leading front of cells was 100 μm or approximately one cell diameter into the gel (Figure 4B, H100). The human breast cancer cell lines invaded into type I collagen with the leading front of MCF-7 cells approximately six cell diameters into the gels and the leading front of MDA-MB-231 cells was 12 cell diameters into the gels (Figure 4B, MCF7 and M231).

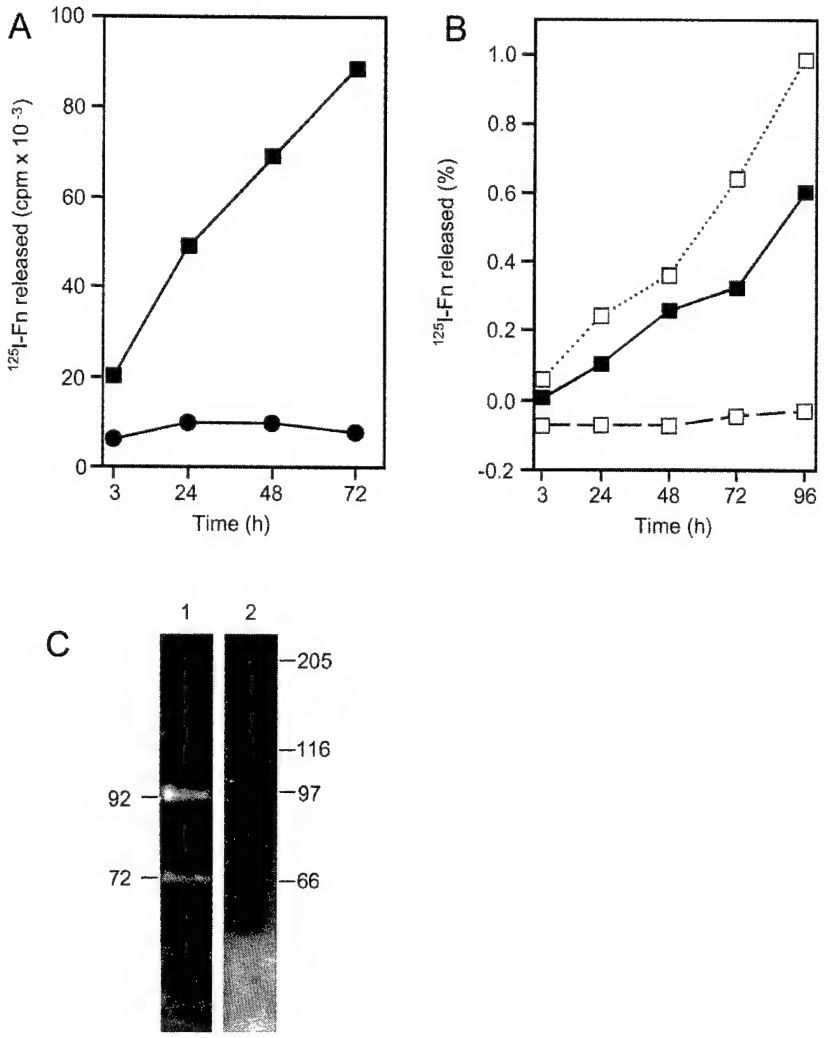

Figure 3. Quantification of matrix proteolysis by normal and malignant human breast cells. (A) Malignant MDA-MB-231 human breast cells (■) release up to 11-fold more fibronectin into the media than the normal HBL-100 human breast cells (●) over 72 h. Levels of radioactivity were determined in two separate experiments. The average of the two determinations after background subtraction is plotted at each time point. (B) Inhibition of MDA-MB-231 human breast cancer invadopodial proteolysis of matrix by the metalloproteinase inhibitor batimastat. Batimastat-treated (10 μM) MDA-MB-231 human breast cancer cells (-□-) release less [^{125}I]fibronectin from the substrate than the no-cell control (background subtracted). MDA-MB-231 human breast cancer cells degrade [^{125}I]fibronectin in the absence (... □ ...) or presence of the DMSO (■) vehicle. Results are expressed as percentage of substrate released and plotted to the average of determinations made in two separate experiments. The average of the two determinations after background subtraction is plotted for each time point. (C) Lane 1 is a zymogram showing gelatinase activities at 92 and 72 kDa secreted into serum-free growth medium conditioned by MDA-MB-231 cells. Lane 2 is a zymogram of the same sample incubated with 10 μM batimastat causing loss of the 92 and 72 kDa gelatinase activities.

Batimastat inhibits MDA-MB-231 human breast cancer cell invasion into type I collagen gels

The correlation between invadopodial matrix-degrading activity (Figures 1–3) and invasion into type I collagen gels (Figure 4) suggested that inhibition of invadopodial matrix proteolysis would decrease invasiveness of the malignant cells. To substantiate the observed correlation, the effect of inhibiting invadopodial proteolysis of matrix on invasiveness was investigated. Batimastat inhibits invasion of MDA-MB-231 cells into type I collagen gels (Figure 5A). Batimastat inhibited invasion from 2.7-fold to 10-fold relative to the percentage of invading cells observed in control cultures growing in the presence of DMSO (Figure 5A). Malignant MDA-MB-231 cells growing in growth medium or those growing in growth medium with added DMSO revealed large invading cell populations (Figure 5A). Normal HBL-100 cells had low numbers of cells invading the type I collagen gels (Figure 5).

Batimastat also reduced the leading front of invasion observed for MDA-MB-231 human breast cancer cells (Figure 5B). Batimastat caused the leading front of invasion to be indistinguishable from cells at the top of the gel or located less than half as deep as that observed for cells grown without batimastat (Figure 5B). MDA-MB-231 breast cancer cells growing in growth medium, or in growth medium with added DMSO revealed leading fronts of invasion ranging from 9 to 11 cell

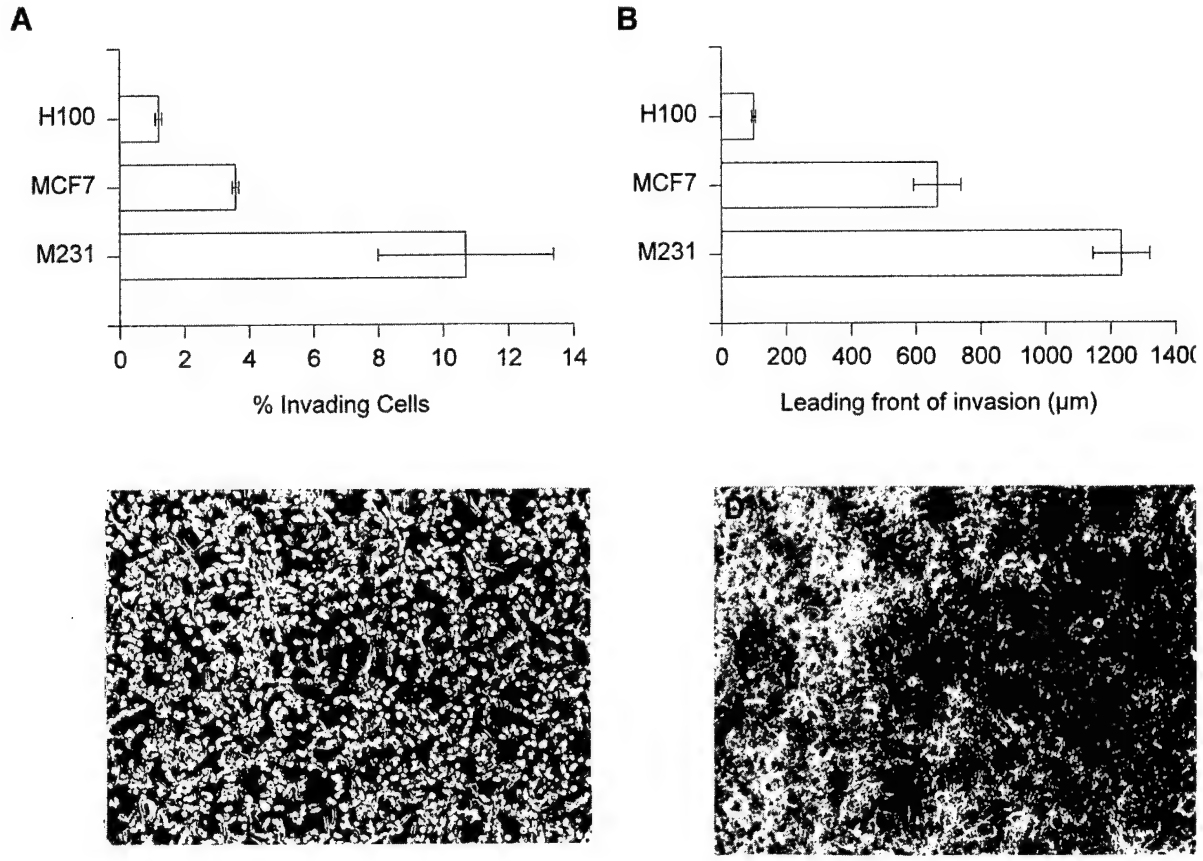

Figure 4. MDA-MB-231 human breast cancer cells invade into type I collagen gels. (A) Invasion of different human breast cell lines into type I collagen gels. The percentage of cells that invade into type I collagen after 72 h was determined for three different human breast cell lines. The HBL-100 normal human breast cell line (H100) is least invasive with only 1.2% ± 0.1 invading cells, the MCF-7 human breast cancer cell line (MCF7) is moderately invasive with 3.58%± 0.4 invading cells, and the MDA-MB-231 human breast cancer cell line (M231) is most invasive with 10.7% ± 2.7 invading cells. In these experiments, cells were grown in medium containing 10% complete FBS. For each cell line, the bar length indicates the average value of 16 different determinations obtained in eight separate experiments. Error bars indicate ± the standard error of the mean. (B) Depth of the leading front of invasion into type I collagen gels of different human breast cell lines. The depth of the leading front of cells invading into type I collagen gels was determined after 72 h for three human breast cell lines. The HBL-100 cells (H100) invade least deeply into the type I collagen gels (100 μm, ± 5.8), MCF-7 cells (MCF7) invade to a moderate depth (667 μm, ± 72.7) and MDA-MB-231 cells (M231) invade furthest into the type I collagen gels (1.233 μm, ± 88.2). For each cell line, the bars indicate the average value of 40 determinations obtained in the same eight experiments reported in (A). Error bars are drawn to ± the standard error of the mean. (C) MDA-MB-231 human breast cancer cells growing on top of a type I collagen gel. Cells (5×10^4) were seeded on top of the gel and allowed to invade for 72 h. In this panel, the plane of focus is the top of the gel. (D) Three MDA-MB-231 cells (arrows) are shown invading 600 μm into the type I collagen gel. They were located by focusing down from the top of the gel in the microscopic field shown in (C). The scale bar (100 μm) for (C) and (D) is shown in (D).

diameters into the gel (Figure 5B; M231 and M231 + DMSO). Normal HBL-100 cells invaded approximately 2.5 cell diameters into the type I collagen gels (Figure 5B, H100). Together, these results indicate that proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by MMPs.

Discussion

Invadopodial proteolysis of extracellular matrix greatly facilitates invasion by human breast cancer cells. Two main lines of evidence support this conclusion. First, the invasion potential of breast cancer cells is directly correlated with the extracellular matrix degrading activity of their invadopodia.

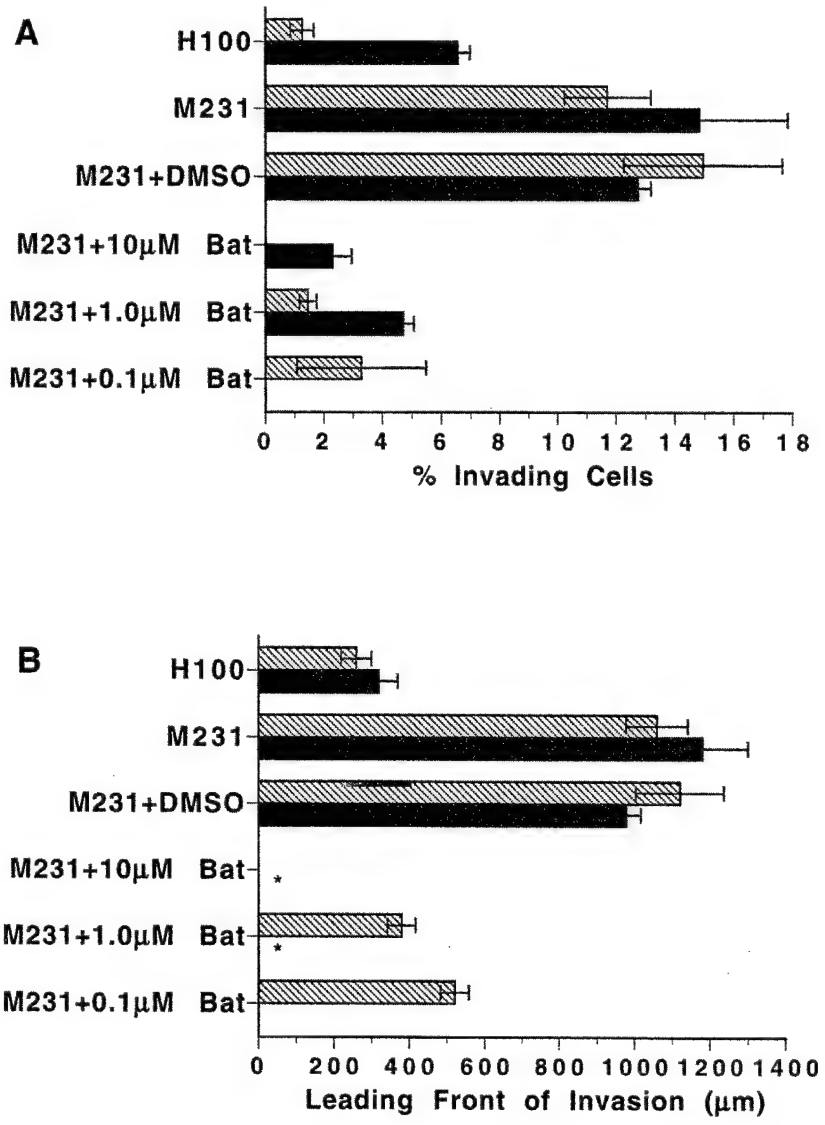

Figure 5. Batimastat inhibits the invasive behavior of MDA-MB-231 human breast cancer cells. The percentage of HBL-100 cells (H100) and MDA-MB-231 cells (M231) invading into type I collagen gels (A) and the leading front of invading cells (B) observed in two different experiments are shown (A, B: hatched bars = experiment 1, black bars = experiment 2). (A) Batimastat inhibits invasion of MDA-MB-231 breast cancer cells into type I collagen gels. Batimastat (1.0 μM) inhibited invasion up to 10-fold relative to DMSO control. Increased batimastat concentrations resulted in decreased invasion (experiment 1 (hatched bars): M231 + 0.1 μM Bat and M231 + 1.0 μM Bat; experiment 2 (black bars): M231 + 1 μM Bat and M231 + 10 μM Bat). The graphs are the average of two data points and the bars indicate the range of the determinations. (B) Batimastat reduces the distance traveled into the type I collagen gel by cells at the leading front of invasion. In experiment 1 (hatched bars), the leading front of invasion was reduced 2.2-fold by 1.0 μM batimastat (M231 + 1.0 μM Bat) and 2.9-fold by 0.1 μM batimastat. In experiment 2 (black bars), invading cells were not detected (*) below the top of the type I collagen gel in the batimastat-treated groups (M231 + 10 μM Bat* and M231 + 1.0 μM Bat*). The graphs are averages of measurements of the leading front of invasion at five locations within the gel and bars represent ± standard error of the mean.

For each cell line tested, the relative level of invadopodial proteolysis of extracellular matrix as measured using fluorescent or radiolabeled fibronectin substrates, positively correlated with each of two different measurements of invasiveness: (i) the percentage of cells invading into type I collagen gels and (ii) the distance traveled by cells at the leading front of invasion. Second, treatment of invasive MDA-MB-231 human breast cancer cells with the MMP inhibitor, batimastat, reduced invadopodial function as assessed by measuring cell-mediated proteolysis of immobilized fibronectin. Batimastat also reduced MDA-MB-231 cell invasion into type I collagen gels as reflected by the reduced percentages of invading cells and shorter distances invaded into the gels relative to untreated cells. Batimastat inhibition of invadopodial matrix proteolysis and invasion is apparently due to its inhibition of MMP proteolytic activities because batimastat effectively inhibited two gelatinase activities at 92 and 72 kDa that were secreted by the MDA-MB-231 cells. However, batimastat did not affect cell viability. The low cytotoxicity of batimastat and MMP inhibition by batimastat over the range of concentrations used in this study (0.1–10 μm) has also been observed for human MDA-MB-435 breast cancer cells [28]. The results presented here confirm separate observations regarding either invadopodial proteolysis or invasion of human breast cancer cells reported by others [8,17]. This study strengthens the linkage of

invadopodial proteolysis of extracellular matrix to the invasion process because both parameters were observed and manipulated in the same study.

MMPs play a critical role in mediating the invadopodial proteolysis of extracellular matrix that facilitates tumor cell invasion. This is evidenced by the effectiveness of the MMP inhibitor batimastat in simultaneously reducing invadopodial proteolytic activity and invasiveness of malignant breast cells. A number of MMPs have been implicated in having a role in proteolysis of extracellular matrix by human breast cancers including: MMP-2 (gelatinase A) [29, 30], MMP-9 (gelatinase B) [2, 31, 32], MMP-11 (stromelysin-3) [33], an 80 kDa MMP [34] and MMP-14 (MT1-MMP) [35]. Batimastat inhibition of MMPs has been effective in reducing the growth and spread of mammary tumors in animal models [28, 36]. Moreover, batimastat and a more bio-available derivative of batimastat called marimastat (BB-2516) are being evaluated in clinical trials for use as an anti-tumor therapeutics [37, 38]. The development of MMP inhibitors for use in anti-tumor therapies continues to be a promising area of research [26].

Although there is mounting evidence that MMPs have a critical role in tumor cell invasion into the complex basement membrane and stromal matrices within living organisms, the evidence also suggests that coordinated activity of other proteases and glycosidases in addition to MMPs is needed to efficiently degrade the matrix and enable invasion [1]. This study was primarily focused on the role of MMPs in breast cancer cell invasion because the gel substrate used, triple helical type I collagen, is resistant to proteolytic cleavage by other classes of proteases [2]. However, an *in vitro* study showed that in the presence of serum, batimastat only inhibited MDA-MB-231 proteolysis of human endothelial basement membranes by 30%. An additional 30–40% inhibition of basement membrane degradation was achieved when batimastat was used in combination with inhibitors of urokinase-type plasminogen activators [39]. There is an apparent need for other classes of proteases for tumor cells to efficiently degrade complex extracellular matrices.

Elucidation of the mechanisms that concentrate MMPs and other extracellular matrix-degrading proteases to invadopodia may lead to new malignant cell diagnostics and strategies to inhibit their invasion. MMP-2 and other matrix-degrading proteases are not restricted to invadopodia. MMP-2 is secreted as a soluble enzyme that can be found within the cytoplasm of cells and embedded in the extracellular matrix as well as on invadopodia. The integral membrane MMP, MT1-MMP, has been identified as an activator of latent MMP-2 and proposed to serve as a cell surface receptor for MMP-2 [40]. In addition, the $\alpha_v\beta_3$ integrin has also been shown to serve as a cell surface receptor for MMP-2 [41]. MT-MMPs, integrins or both of these molecules may have a role in concentrating MMP-2 to invadopodial membranes of human breast cancer cells. It has been shown that MT1-MMP must be localized to invadopodia to stimulate the local degradation of extracellular matrix that is characteristic of invadopodia and that its cytoplasmic domain has a role in directing MT1-MMP to invadopodial membranes [12]. Another mechanism for recruitment of active proteases to invadopodial membranes could involve directed oligomerization of the subunits of integral membrane proteases such as seprase, fibroblast activation protein-α, dipeptidyl peptidase IV, and meprin [3]. This directed oligomerization might occur in response to signaling through integrins [42]. Perturbing the recruitment and assembly of proteases on the invadopodial surface might effectively reduce invadopodial proteolysis of extracellular matrix and limit invasion.

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Evaluation of seprase activity

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Abstract

Seprase is a serine protease that is integral to the plasma membrane and is overexpressed by invasive tumor cells (Piñeiro-Sánchez et al., J Biol Chem 1997; 272: 7595–601; Monsky et al., Cancer Res 1994; 54: 5702–10). Seprase activity is most often assessed by zymography, which is not a quantitative assay. This study establishes a relatively simple and quantitative method for determining seprase activity. The degradation of a ^{3}H-gelatin substrate is measured in the presence of 5 mM EDTA which inhibits matrix metalloproteinases but not seprase. The quantitative character of the assay was demonstrated using partially purified seprase from chicken embryos, a preparation that lacks detectable matrix metalloproteinase activity. In this assay, release of ^{3}H-gelatin fragments is linear over time for 1.5 μg/assay seprase concentration as well as for preparations concentrated or diluted by five fold (7.5 μg/assay and 0.3 μg/assay respectively). Additional experiments were performed to validate the quantification of seprase activity using the radiographic assay by comparing the results to zymography. Exposure to 22 or 37 °C results in maximal seprase activity while exposure to 80 or 100 °C completely abolishes seprase activity in both zymography and the radiographic assay. Exposure to 60 °C abolished seprase activity as judged by zymography, but about 50% gelatinase activity was observed using the ^{3}H-gelatin substrate. Immunopreciptiation with seprase-specific antibody specifically removed seprase and lowered the seprase activity remaining in the extracts as judged by both assays. Investigation of the seprase that was partially purified from human breast cancer tissue revealed that its specific activity (cpm gelatin fragments released/ {mg protein × h}) is five times greater than that of seprase purified from chicken embryos. This assay will be useful for determining the seprase activity in extracts of tumor tissues and cells as well as for identifying inhibitors of seprase.

Introduction

Seprase was first identified as a 170-kDa gelatinase activity in detergent extracts of the human melanoma cell line LOX [1] and then as a 160-kDa gelatinase activity in detergent extracts of Rous sarcoma virus transformed, chicken embryo fibroblasts [2]. Because seprase partitions to the detergent phase of Triton X-114 extracts, it was determined that seprase is an integral membrane protease [1, 2]. The gelatinase activity of seprase was shown to be sensitive to heat, acid pH, phenyl methyl sulfonyl fluoride (PMSF) and N-methyl maleimide (NEM) but insensitive to β-mercaptoethanol, dithiothreitol, EDTA, 1,10 phenanthroline, pepstatin, and leupeptin; suggesting that seprase is a serine or cysteine protease [1, 2]. The 170-kDa active enzyme is a complex of two identical 97-kDa subunits [3, 4]. The molecular cloning and analysis of a cDNA encoding the human melanoma 97-kDa seprase subunit protein indicate that it is a type II transmembrane serine protease with the catalytic triad amino acid residues (S, D, H) arranged in the non-classical orientation [4, 5]. Interestingly, the seprase subunit protein is proteolytically inactive despite the fact that it includes the entire catalytic domain. Seprase, apparently, requires oligomerization into dimers for proteolytic activity [4, 6]. Inactivation of the seprase proteolytic activity is caused by agents that dissociate the subunit proteins such as acid pH, heat, and alkylating agents including diethyl pyrocarbonate (DEPC) and NEM, as well as by agents that modify the catalytic site, serine, such as PMSF and diisopropyl fluorophosphate (DFP) [4].

Seprase is thought to have a role in facilitating tumor cell invasion and metastasis [1, 6]. Several observations support this hypothesis. First, seprase degrades extracellular matrix and is overexpressed by invasive tumor cells. For example, high levels of seprase activity were detected in extracts of the highly invasive human melanoma cell line LOX , but seprase activity was not detected in extracts of 32 other tumor cell lines that were not invasive in *in vitro* assays [1]. Seprase over-expression by invasive tumor cells has also been observed in pathologic specimens of human breast cancer tissue by immunohistochemistry [7]. Second, seprase is localized on the plasma membrane where it could interact with extracellular matrix substrates, and it is concentrated

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on invadopodia, specialized protrusions of the plasma membrane that cause proteolysis of extracellular matrix [2, 3]. Thirdly, a positive correlation between the level of seprase expression and invasive behavior of LOX human melanoma cells has been observed [3]. Although overexpression of seprase is apparently an important feature of the invasive phenotype, methods for determining seprase activity are not well developed.

Seprase in extracts of cells and tissues has been analyzed by immunoblotting and gelatin zymography, techniques that are difficult to quantify and may not give accurate indications of the level of seprase activity. In addition, immunoblot analysis measures the level of 170-kDa and 97-kDa proteins independent of their proteolytic activities. Zymography is not generally used for quantification of proteolytic activity due to the saturation of the signal that occurs. Zymography and immunoblotting allow identification of seprase in crude extracts but require solubilization of the extract proteins in SDS followed by SDS-PAGE. These treatments can change the apparent activity of proteases [8]. It is conceivable that zymography could reduce the apparent seprase activity by causing dissociation of some assembled dimers into inactive monomers.

This study was performed to establish a quantitative assay for the proteolytic activity of seprase in extract samples. A seprase activity assay was developed that is based on established procedures for measuring the gelatinase activities of MMPs [9]. A ^{3}H-gelatin substrate was used in conjunction with buffers that included EDTA to promote seprase activity while inhibiting the activities of MMPs. The assay is shown to be linear over several concentrations of seprase. This assay will be useful for determinations of the net seprase activity in relatively crude extracts as well as for direct determinations of the activity of purified seprase.

Materials and methods

Sources of seprase

The partial purification of seprase has been described [7] and is based on published methods [1, 4]. Briefly, 9 day-old chicken embryos were rinsed 3 times with cold PBS, and used immediately or stored at −80 °C. The embryos were homogenized and peripheral membrane proteins were removed by sequential extractions. The homogenate was extracted at 37 °C with 10 volumes of 10 mM Tris, 5 mM EDTA, pH 7.6. The insoluble material was collected by centrifugation at 10 000 × *g* and extracted with 10 mM Tris-HCl, 0.6 M NaCl, 5 mM EDTA, pH 7.6 for 1 h at 4 °C. The insoluble material was collected and extracted with 2.6% Triton X-114, 10 mM Tris-HCl, 5 mM EDTA, pH 7.6. The soluble material was collected by centrifugation as described above and the detergent extract supernatant was recovered.

The detergent extract was phase partitioned [10], and the detergent phase was separated from the aqueous phase by centrifugation. The detergent phase was diluted and loaded at 70 ml/h onto an 80-ml DEAE-cellulose column (Whatman DE52, Whatman Labsales, Hillsboro, OR) equilibrated in 0.5% Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5. The column was washed and then eluted with a continuous 0- to 400-mM NaCl gradient in the equilibration buffer. Fractions with seprase activity were identified by zymography and pooled for purification by WGA agarose. Purification of seprase from 11.5 g of human breast tumor tissue was performed as described for chicken embryo seprase, except that the DEAE cellulose column was omitted.

Zymography. Zymography was performed essentially as described by Heussen and Dowdle [11] with 1 mg/ml gelatin copolymerized into 10% SDS polyacrylamide gel.

Gelatinase activity assay. Gelatin (100 mg, type A porcine skin, 300 bloom, Sigma, St. Louis, MO) in 10 ml borate buffer, pH 9.5 was labeled with 1 mCi ^{3}H-acetic anhydride [12]. The ^{3}H-gelatin in the radiolabeled stock solution was separated from free isotope by gel filtration over an 80 ml Sephadex G-150 column at 22 °C. A broad peak of labeled gelatin began eluting immediately after the void volume and was well separated from the sharp peak of unbound isotope. The ^{3}H-gelatin was mixed 1:2 with neutralized, acid soluble type I collagen (1 mg/ml, Sigma, St. Louis, MO) in 50 mM Tris-HCl, pH 7.6, 5 mM EDTA. The collagen was denatured by heating at 55 °C for 30 min.

The gelatinase activity of seprase was determined using a modification of the assay of Stetler-Stevenson and co-workers [9]. Reactions to determine the gelatinase activity of seprase were performed in triplicate in the wells of a 96 well microtiter plate with 13 μl seprase-containing fractions; 13 μl of ^{3}H-gelatin substrate, and 47 μl of the reaction buffer: 0.1% Triton X-100, 50 mM Tris-HCl, 5 mM EDTA, pH 7.6. Samples to determine the background cpm were prepared by mixing 60 μl of reaction buffer and 13 μl substrate then precipitating the undigested gelatin with 20 μl 10% trichloro acetic acid, 5% tannic acid (TCA/TA) on ice.

Samples to determine the total counts were prepared by mixing 13 μl substrate, with 80 μl of reaction buffer but no TCA/TA was added to these samples. Experimental reactions were stopped at various time points and the undigested substrate precipitated by addition of 20 μl TCA/TA. Reactions were precipitated for 1 h on ice and then the precipitates were cleared by a 10 min, 1200 × *g* centrifugation at 4 °C in a Beckman TJ-6 refrigerated table top centrifuge and a TH-4 rotor. Samples (60 μl) were mixed vigorously in 4.5 ml of Ecolume scintillant (ICN Pharmaceuticals Inc., Costa Mesa, CA) and counted with a Packard 1600 TR liquid scintillation counter.

To determine if the assay was linear over several concentrations, seprase enriched fractions were concentrated 5 fold to 0.58 mg/ml using centricon 30 filter concentrators (Amicon, Inc, Beverly, MA) and then used as is, diluted to the original concentration, or diluted to 1/5 the original concentration with reaction buffer.

Determination of fold purification. The activity for each fraction was determined using the ^{3}H gelatin substrate and the gelatinase activity assay described above. The cpm of

TCA/TA-soluble ^{3}H gelatin liberated per mg protein was determined in triplicate at 1, 5, 10, 24, and 30 h for each fraction, and the average of these determinations was plotted. A line was drawn to pass through the origin and use all available data by first order (linear) regression, as calculated by Jandel Sigma Plot for Windows (SPSS, Inc., Chicago, IL). For each fraction, the slope of the 'cpm/mg' versus 'time' line is equal to the specific activity of the seprase in the sample, and was used to determine the fold purification. Activity determinations have been performed on 3 other seprase preparations with similar results.

Immunoprecipitations. A rat polyclonal antiserum was elicited against affinity purified chicken embryo seprase as described earlier [7]. Immunoprecipitations were performed using 15 μl rat serum directed against the 97-kDa protein or 15 μl of preimmune serum from the same rat, 200 μl protein A agarose (Pierce, Rockford, IL), and 200 μl of WGA-purified chicken embryo seprase. The subtraction of seprase was determined by gelatin zymography. Immunoprecipitations were also performed to measure the effect of removing seprase activity on the gelatinase activity of partially purified seprase preprarations. These precipitations were performed by coating 100 μl of protein A beads with 30 μl or 60 μl of antiserum, washing away unbound proteins, and then exposing the antibody-coated protein A agarose to 200 μl WGA-purified chicken embryo 160-kDa protein. The gelatinase activity present in the fraction that was not bound to the antibody-coated beads was measured by soluble gelatinase activity assay described above. The gelatinase activities of the purified seprase preparation used for these experiments and of the fraction not bound by protein-A beads coated with nonimmune rat antiserum were also determined.

Protein determinations were performed using the bicinchoninic acid assay and bovine serum albumin dissolved in Tris-HCl, pH 6.8, 1% (w/v) SDS as the standard protein (Pierce, Rockford, IL).

Results

A source of seprase was needed to enable development of a quantitative assay for seprase activity. Chicken embryo seprase was partially purified and used to establish a gelatinase activity assay with a ^{3}H-gelatin substrate. The partial purification procedure was developed from previously published methods for purifying human melanoma cell seprase [1, 4]. A series of extractions removes many peripheral membrane proteins and then the seprase is solubilized with Triton X-114 and EDTA. Seprase activity partitions exclusively to the detergent phase and is further purified using DEAE-cellulose and WGA-agarose. Gelatin zymography reveals that the resulting preparation has high 160-kDa seprase activity but no detectable MMP activity (Figure 1). Specifically, the 70-kDa chicken homolog of MMP-2 is not detected in the partially purified seprase preparations (Figure 1). Proteolysis of a ^{3}H-gelatin substrate by this preparation was investigated by incubating the seprase and the ^{3}H-gelatin over time and then using TCA/TA to precipitate the undigested substrate at different time points. Measurement of the TCA/TA-soluble, radiolabeled gelatin degradation products at various time for several different levels of the same fraction of partially purified seprase protein (7.5 μg, 1.5 μg, and 0.3 μg) over 5 h reveals that the observed gelatinase activity increases with increasing concentrations of seprase (Figure 2A). Plotting the slopes of this data (cpm/h) versus protein concentration reveals that these samples are within the linear range of the assay (not shown). Investigation of samples from different steps in the seprase enrichment procedure reveals a close correspondence between the increasing purification of seprase and increasing gelatinase activity (Figure 2B). Moreover, these experiments were conducted over 30 h and the assay remained linear over this time frame (Figure 2B). The gelatinase activity assay reveals a 21.8 fold enrichment of the seprase activity by the purification procedure (Table 1).

Table 1. Enrichment of chicken embryo seprase – 429.4 g chicken embryos.

Fraction	Units activity (cpm/{mg × h})	Fold purification	Protein (mg)
Initial extract	263.9	–	7238
TX-114 extract	265.7	1.0	617.5
Detergent phase	328.1	1.2	328.9
DE-52	3467.9	13.1	14.8
WGA	5742.9	21.8	5.8

Investigation of the temperature sensitivity of chicken embryo seprase reveals that seprase activity is not affected by 15 min preincubation at 22 or 37 °C, because maximal activity is observed by both gelatinase activity assay (Figure 2C) and zymography (Figure 2D). However, a 15-min preincubation of the seprase at 80 or 100 °C inactivates the seprase and no activity is observed in either assay (Figures 2C and D). Preincubation of the seprase at 60 °C partially inactivates the seprase as judged by gelatinase activity assay (Figure 2C) but completely inactivates seprase as judged by zymography (Figure 2D). Thus, the loss of seprase activity by heating is detected as a reduction in gelatinase activity by both assays.

An antiserum that recognizes both active 160-kDa chicken embryo seprase (Figure 3A, Active) and the proteolytically inactive 97-kDa subunit of chicken embryo seprase (Figure 3A, Inactive) was used to immunoprecipitate seprase. Immunoprecipitation with the seprase specific antibody removes much of the seprase and causes a corresponding drop in the seprase activity that remains in the extract. This specific reduction in seprase activity is detected by both zymography (Figure 3B) and gelatinase activity assays (Figure 3C). The antibody to seprase removes up to 50% of the seprase activity from the unbound fraction (Figure 3C, plot D) relative to the unbound fraction of control precipitates (Figure 3C, plot B).

Seprase was also partially purified from human breast tumors (11.5 g) using the procedure described above for

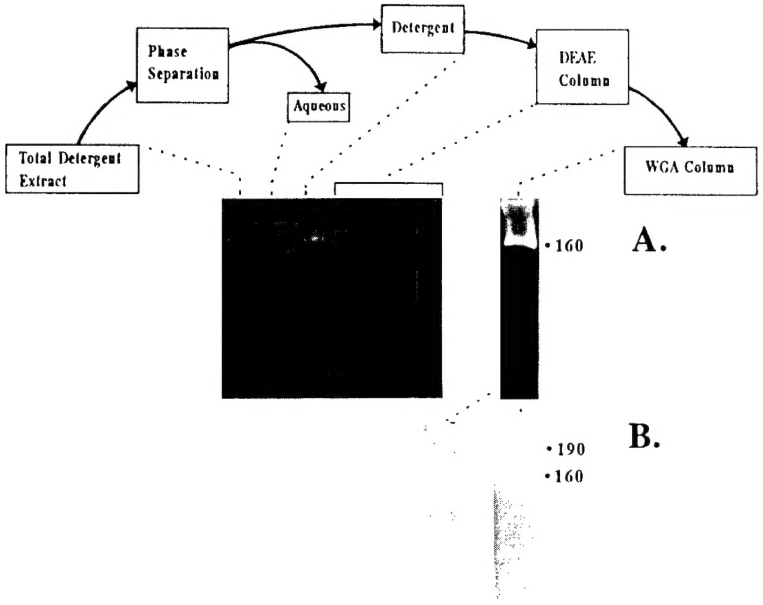

Figure 1. Partial purification of seprase from chicken embryos. (A) The upper flow-chart outlines the purification procedures. The middle panels show the seprase activity in different fractions of chicken embryo seprase purification as detected by gelatin zymography. Seprase activity is extracted by 2.6% Triton X-114 (total detergent extract) and partitions strictly to the detergent phase (detergent). The detergent phase was loaded onto a DEAE cellulose column. Seprase bound to the column and the majority of the activity eluted between 0.1 M NaCl and 0.25 M NaCl (DEAE column). This fraction was loaded onto a wheat germ agglutinin column. The seprase bound to the column, indicating that it is a glycoprotein, and was eluted with 10% (w/v) n-acetyl glucosamine (WGA column upper and lower panels). Molecular weight markers are given on the right $\times 10^{-3}$. (B) The panel on the left is an SDS-PAGE of the proteins in chicken embryo seprase that was partially purified as described above. The panel on the right is a gelatin zymogram of this same sample. Both gel and zymogram were stained with Coomassie Brilliant Blue R-250. Stained proteins are detected at 190 kDa and 160 kDa that commigrate with the gelatinase activity. Molecular weights are given on the right $\times 10^{-3}$.

Table 2. Enrichment of human breast cancer seprase – 11.5 g human breast cancer tumor.

Fraction	Units activity (cpm/{mg × h})	Fold purification	Protein (mg)
Initial extract	1852	–	61.08
WGA	38680	21	0.155

partial purification of chicken embryo seprase, except that the DEAE cellulose column was omitted. These preparations reveal a 170-kDa gelatinase activity that is seprase because: i) the gelatinase is active in the presence of EDTA which inhibits MMPs, ii) the gelatinase activity requires detergent for solubilization and partitions to the detergent phase of Triton X-114 extracts (Figure 4), and iii) the gelatinase binds to WGA. Seprase was the only gelatinase activity detected by zymography in these samples (Figure 4). The gelatinase activity assay revealed that human breast cancer seprase is purified 21 fold by this procedure (Table 2). Moreover, breast cancer seprase is 5 times more active than chicken embryo seprase when the specific activities (cpm gelatin released/{mg protein × h}) of seprase from the two sources are compared (Tables 1 and 2).

Discussion

Seprase activity in extracts can be measured using a ^{3}H-gelatin substrate. This conclusion is supported by the close correlation of the relative seprase gelatinase activities in different samples as judged separately by the gelatinase activity assay and zymography. Specifically, loss or reduction of seprase activity by heating or immunoprecipitation resulted in corresponding losses or reductions in the observed seprase band of activity on zymograms and amount of soluble radiolabeled gelatin fragments. Concentrating or enriching seprase activity resulted in corresponding increases in seprase activity in both assays. One difference between zymography and the seprase activity assay was that after heating to 60 °C seprase activity was observed in the gelatinase activity but not by zymography. This result likely reflects the re-naturation and re-association of a fraction of the seprase subunit proteins during the gelatinase activity assay. In zymography, such reassociation of seprase subunits probably would not occur due to SDS in the gel sample buffer binding to dissociated seprase subunits and the subsequent separation of the proteins by SDS-PAGE. The gelatinase activity assay allows the amount of apparent seprase activity to be determined relative to extract protein. In this way, the apparent specific activity of seprase was observed to be 5 times greater in extracts of human breast cancer tissue than in chicken embryo extracts. This finding is consistent with the overexpression of seprase in breast cancer tissues observed by immunohistochemistry [7] and

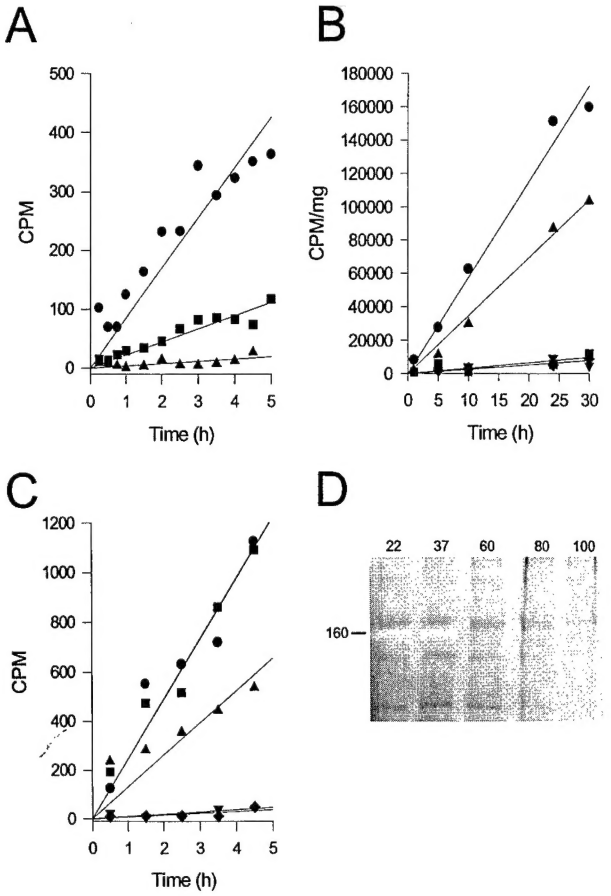

Figure 2. Quantification of the gelatinase activity of seprase (A) The seprase activity assay is linear over a 5-h time period using a ^{3}H-gelatin substrate and three concentrations of the same partially purified chicken embryo seprase fraction: 7.5 μg (●), 1.5 μg (■) and 0.3 μg (▲). The TCA/TA-soluble ^{3}H-gelatin proteolyltic fragments were determined (CPM) over five hours (time (h)). Gelatinase activity increases with increasing concentration of seprase. Assays were performed in triplicate in the presence of 5 mM EDTA, 0.1% Triton X-100 and each point is the average of 3 assays after background subtraction. For each plot, the solid line represents the first order regression of the data. (B) Fractions from the purification of chicken embryo seprase were analyzed by gelatinase assay to determine the substrate hydrolyzed per mg of protein (CPM/mg) over time (h). The plots are the first order regressions for: the initial low ionic strength extraction (▼); total detergent extract (♦); detergent phase (■), DEAE pooled fractions (▲), and WGA pooled fractions (●). Observed gelatinase activities increase with increasing seprase purity. Assays were performed in triplicate in the presence of 5 mM EDTA, 0.1% Triton X-100 and each point is the average of 3 assays after background subtraction. (C) Chicken embryo seprase (7.5 μg) was maintained at temperatures of 22, 37, 60, 80, or 100 °C for 15 min and then used for gelatinase assay. Seprase was not affected by incubation at 22 (●) or 37 °C (■), as the observed gelatinase activities are identical (first order regression for both sets of data results in superimposed lines). Gelatinase activity was significantly reduced by treatment at 60 °C (▲), and destroyed by 80 (▼) or 100 °C(♦). Assays were performed in triplicate in the presence of 5 mM EDTA, 0.1% Triton X-100 and each point is the average of 3 assays after background subtraction. (D) By gelatin zymography, seprase activity is detected only in samples treated with 22 and 37 °C. The activity is lost in samples exposed to 60, 80 and 100 °C. Molecular weight is given $\times 10^{-3}$.

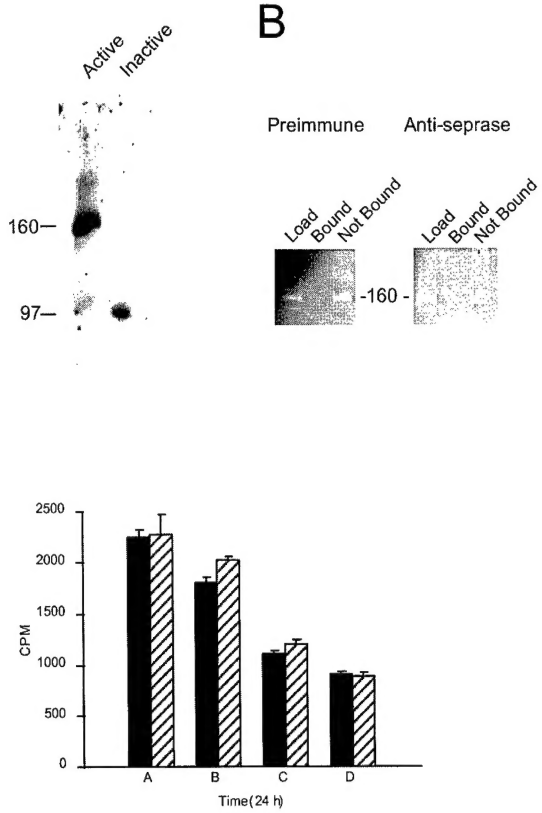

Figure 3. The gelatinase activity assay determines specific reductions in seprase activity. (A) Immunoblot analysis of WGA-purified chicken embryo seprase. A polyclonal antibody to seprase recognizes the 160-kDa active seprase dimer together with a low level of a 97-kDa subunit protein (active). Boiling this sample prior to electrophoresis results in loss of the 160-kDa protein and increased 97-kDa protein (inactive). (B) Immunoprecipitation of the seprase-gelatinase activity as determined by zymography. Gelatin zymogram showing that chicken embryo seprase activity (white band at 160 kDa, both panels) in the original extract (load lanes, both panels) is not significantly reduced by immunoprecipitation with preimmune serum (preimmune/not bound). However much of the seprase activity is removed by immunoprecipitation with the antibody against seprase because much less activity is present in the unbound supernatant fraction (not bound/anti-seprase) relative to that in the initial extract (load/anti-seprase). Molecular weights are given $\times 10^{-3}$. (C) Immunoprecipitation of seprase-gelatinase activity as determined by an activity assay using a ^{3}H-gelatin substrate. Gelatinase activity in a seprase preparation used for immunoprecipitation experiments (A). Non-immune rat IgG did not precipitate seprase activity and the activity remaining in the unbound fraction (B) is only slightly reduced relative to that in the initial extract. Seprase activity was specifically removed from the extract by immunoprecipitation with 30 μl (C) or 60 μl (D) rat polyclonal antibody against seprase, resulting in a reduction in the gelatinase activity remaining in the extract relative to the initial extract. Two separate experiments are shown (black & hatched bars) with bar height indicating the average of the three determinations. Error bars are drawn to the standard error of the mean.

suggests that breast cancer tissue has a high level of fully assembled, active seprase. It is also consistent with the differential expression of seprase in chicken embryonic tissues because the initial extract includes proteins from the entire embryo where some tissues have detectable seprase activity and others do not (V. Kaushal; L. A. Goldstein; W.-T. Chen and T. Kelly, manuscript in preparation). However, the possibility that the breast cancer extracts contain additional

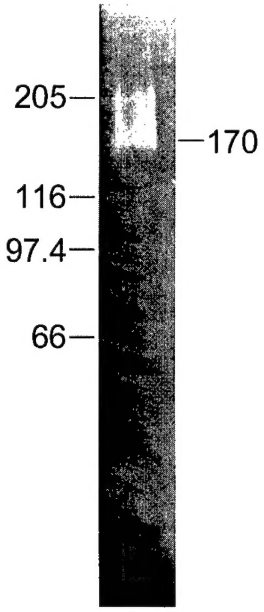

Figure 4. Seprase activity is present in extracts of malignant human breast tumors. Seprase was enriched in the detergent phase of Triton X-114 extracts of human tumors that were evaluated by pathologists to be infiltrating ductal carcinomas of the breast. Seprase activity at 170 kDa and smearing to 205 kDa is apparent in this fraction as judged by zymography. Molecular weight markers are given $\times 10^{-3}$.

proteases that are not detected by zymography or reduced inhibitors of these proteases can not be completely ruled out.

Determination of seprase activity in extracts at physiologic pH and salt concentrations affords a means to investigate seprase activity under conditions that more closely resemble those found *in vivo*. Quantitative comparisons of activity can be made between different samples with the determinations focused on the active enzyme and not the level of inactive subunit proteins. The technique will be useful for measuring the level of seprase activity of cells that overexpress seprase as part of pathologic processes or because they have been genetically engineered for seprase overexpression. The seprase activity assay may be particularly useful for identification of molecules that inhibit seprase activity. Antibodies and other non-toxic inhibitors of seprase will be valuable for clarifying the role of seprase in tumor cell invasion and may ultimately have clinical utility.

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